REMARKS

A check for the fee for a two month extension of time accompanies this response. Any additional fees that may be due in connection with filing this paper or with this application during its pendency may be charged to Deposit Account No. 06-1050. If a Petition for extension of time is required, this paper is to be considered such Petition.

Amendments to the Claims

Upon entry of the above amendment, claims 8, 10, 11, 13, 16, 18-21, 23-25, 27 and 32-36 will be pending in this application. In the interest of placing the application in condition for allowance or reducing the number of issues for appeal, claim 8 is amended and claims 9, 17, 22, 26 and 29-31 are canceled. Applicant reserves the right to file continuation and/or divisional applications directed to any cancelled and/or unclaimed subject matter.

Claim 8 is amended to recite an isolated nucleic acid encoding a polypeptide of SEQ ID NO:1. Basis for this amendment can be found in the specification, for example, in original claim 9 and at page 6, lines 6-8. Claims 34 and 35 are amended to correct an error in their dependency. No new matter is added.

Withdrawn method claims 33-35, which incorporate the limitations of product claim 8, are retained for possible rejoinder if product claim 8 is deemed allowable, in accordance with MPEP §821.04.

I. INTERVIEW WITH THE EXAMINER ON JANUARY 10, 2008

The amendments and arguments provided with this response are in accordance with discussions during an interview with the Examiner that took place on January 10, 2008. Applicant thanks the Examiner for the courtesy extended in granting an interview to Applicant's representatives, Dr. Janis Fraser and Dr. Jayshree Gerken. Addressed during the interview were the following: (1) The rejection of claims 8-11, 13, 16-25, 27 and 29-32 as allegedly lacking either a well-established utility, or a specific, substantial and credible asserted utility; and (2) The rejection of claims 8-11, 13, 16-25, 27 and 29-32 as allegedly lacking enablement, insofar as the rejection pertained to variants of the human OCTN1 polypeptide that are encompassed by the claims.

(1) With regard to lack of utility, Applicant's representatives pointed out that the claimed isolated nucleic acids encode a protein that belongs to a class of proteins, organic cation transporters, which are long well-established as being involved in mediating and

regulating drug uptake and distribution. Applicant's representatives further pointed out that as an asserted utility in the specification, the use of the encoded human OCTN1 (hOCTN1) organic cation transporter to screen for carcinostatic compounds that might best be taken up by cancer cells and tissues in which the protein is expressed is specific, *i.e.*, not generically applicable to any protein, and substantial, *i.e.*, it has "a significant and presently available benefit to the public" (In re Fisher, 421 F.3d 1365, 76 USPQ2d 1225 (Fed. Cir. 2005)). The Examiner did not question the credibility of the asserted utility, hence this factor was not addressed.

The Examiner responded that Applicant's arguments were not persuasive because in the absence of information about a physiological role for the encoded human OCTN1 protein, no "nexus" between the protein and a disease can be established, thus providing no basis for targeting the OCTN1 protein as a way of treating a disease. The Examiner further stated that in the absence of an understanding of a physiological role for hOCTN1, the utility of the hOCTN1 protein in an assay to screen for carcinostatic compounds amenable to being transported by the protein was neither specific nor substantial. The Examiner alluded to recent unpublished decisions of the Board of Patent Appeals and Interferences as being consistent with her position, but was unable to cite specific cases. Applicant's representatives then requested an interview with the Examiner's supervisor to further discuss the rejection, and the Examiner responded that she would try to arrange for the same.

On January 17, 2008, Applicant's representative Dr. Jayshree Gerken made a follow-up telephone inquiry to the Examiner regarding the availability of her supervisor for an interview. The Examiner responded that it likely would not be possible to arrange for an interview in the month of January, due to the supervisor's busy schedule.

The supervisor did call Dr. Gerken on January 23, 2008, and left a voice message informing her that he supported the Examiner's position regarding lack of utility. He suggested that Applicant schedule a pre-appeal conference to discuss the utility issue. On January 25, 2008, Dr. Gerken called the Examiner and the Examiner's supervisor to request consideration and entry of an Amendment after Final, at least to the extent it could reduce the number of issues for appeal by satisfactorily addressing the rejection on grounds of lack of enablement (see (2) below). The Examiner responded that consideration and entry of an Amendment after Final was possible, to the extent it reduces the number of issues for appeal. On January 28, 2008, the Examiner's supervisor confirmed the same.

Applicant, therefore, is submitting this Amendment after Final for consideration and entry. As discussed with the Examiner on January 25, 2008, the Amendment after Final summarizes Applicant's traversal of the rejection on grounds of lack of utility. In addition, Applicant has addressed the enablement rejection in a manner that should reduce the number of issues for appeal, as discussed during the interview of January 10, 2008 and below.

(2) With regard to the enablement rejection as it pertains to variants of human OCTN1, Applicant's representatives proposed that in the interest of placing the application in condition for allowance or reducing the number of issues for appeal, claims that recite or are directed to nucleic acids encoding proteins whose sequence is 70%, 76%, 80% or 90% identical to the human OCTN1 amino acid sequence (SEQ ID NO:1), would be amended or canceled as appropriate to remove reference to these variants. Applicant however proposed retaining claims 10 (and its dependent claims 18 and 23), which specify that the nucleic acid has one to thirty conservative amino acid substitutions in the human OCT N1 sequence (total length 551 amino acids), and claim 32, which specifies one to ten conservative amino acid substitutions, as variants that could reasonably be expected to preserve the function of human OCTN1. The Examiner agreed to reconsider this ground of rejection as applied to claims 10, 18, 23 and 32.

II. THE REJECTION OF CLAIMS 8-11, 13, 16-25, 27, 29-32 AND 36 UNDER 35 U.S.C. §101 - UTILITY

Claims 8-11, 13, 16-25, 27, 29-32 and 36 are rejected under 35 U.S.C. §101 as allegedly lacking a well-established utility or a credible, substantial and specific asserted utility. With respect to the asserted utilities in the specification, the Examiner goes on to allege that while their credibility is not at issue, the utilities all lack specificity and are not substantial.

In the previous response filed February 23, 2007, Applicant described various asserted utilities as being specific, substantial and credible, including the use of the organic cation transporter proteins encoded by the claimed nucleic acids, human OCTN1 and variants containing from one to thirty conservative amino acid substitutions, to screen for carcinostatic agents that are amenable to being transported by these proteins. In the instant Final Office Action, the Examiner rebuts Applicant's arguments for a variety of reasons, exemplary of which are the following:

At pages 9-11 of the Final Office Action, the Examiner, citing the post-filing reference Tamai *et al.* (*FEBS Letters* 419:107-111, 1997; of record in the instant application's file history), alleges that the asserted utilities for developing carcinostatics, or for screening for carcinostatic compounds or other compounds whose absorption is mediated by human OCTN1 (hOCTN1), are neither specific nor substantial because as Tamai *et al.* apparently notes, although hOCTN1 was found in several cancer cell lines and in some normal tissues, its physiological role was not established. The Examiner goes on to state that although hOCTN1 is expressed in a few cancer cell lines, there is no evidence that this expression is altered in cancer cells compared to normal cells and therefore there is "nothing specific or substantial" for the compounds screened for transport mediated by hOCTN1. The Examiner acknowledges that the hOCTN1 protein transports carcinostatics, but states that this observation is neither specific nor substantial because, in the absence of a physiological role for hOCTN1, the transport screening assays can allegedly be performed "with any polypeptide and nucleic acid." The Examiner opines that the instant situation is analogous to the facts of Brenner v. Manson, 148 U.S.P.Q. 689 (Sup. Ct. 1966) because like the chemical compounds in Brenner, the claimed nucleic acids encoding organic cation transporter proteins allegedly have utility only in the broadest sense and not "in an immediately obvious or fully disclosed real world utility."

During the interview of January 10, the Examiner again communicated her view that *any* utility of a newly discovered protein like hOCTN1 (or nucleic acid encoding the protein), barring a known physiological role, or a well-characterized nexus between the protein and a disease state, is neither specific nor substantial. She said that she based her assertion on several recent unpublished decisions by the Board of Patent Appeals and Interferences, but could not provide specifics.

This rejection is respectfully traversed. The rejection is rendered moot with respect to claims 9, 17, 22 and 29-31, which are cancelled herein. Applicant's response to the previous Office Action, filed February 23, 2007, including the arguments addressing the utility rejection, are incorporated in its entirety by reference herein.

Analysis

Applicant submits that the specification asserts at least one credible, specific and substantial utility, namely, screening for carcinostatic compounds that are transported by hOCTN1 (*see* specification, for example, at page 31, lines 5-16). Applicant further submits

that the asserted utility is well-established; *i.e.*, even had it not been explicitly asserted in the specification, it nonetheless would have satisfied the utility requirement.

1) Asserted Utility that is Specific, Substantial and Credible

The claims are directed to isolated nucleic acids encoding polypeptides having the amino acid sequence set forth in SEQ ID NO:1 (hOCTN1) or certain variants thereof, expression vectors and cultured cells containing these nucleic acids, methods of producing the encoded polypeptides from the cultured cells, and methods of screening for compounds, including carcinostatic compounds, that are transported by polypeptides encoded by the isolated nucleic acids.

The specification teaches that the encoded hOCTN1 polypeptide contains the structural features characteristic of a well-known class of proteins, organic cation transporters. The specification further teaches that when the encoded protein is expressed in cells, it in fact performs the function of an organic cation transporter. The working examples demonstrate hOCTN1-mediated transport of a variety of compounds, including carcinostatics such as actinomycin D, etoposide, vinblastine and daunomycin. The specification also teaches the distribution of the hOCTN1 organic cation transporter in a variety of cells and tissues, including its prevalence in tumor cell lines.

Based on the teachings as a whole, as discussed above, the specification asserts a utility for the claimed isolated nucleic acids, namely, expressing the encoded hOCTN1 transporter proteins in cells and screening for carcinostatics that are preferentially absorbed and transported by hOCTN1. The screen permits the selection of carcinostatics that will preferentially be absorbed by target tissues or cells that express the hOCTN1 transporter. If one is trying to treat a cancer in which the cells express the hOCTN1 transporter, one can use the screen to identify which of a number of potential carcinostatics would be most effectively taken up by the cells. The asserted utility is <u>specific</u> because it is particular to the type of protein, the hOCTN1 organic cation transporter, discovered by Applicant. Contrary to the Examiner's assertions, the screen is not broadly applicable to any protein, it is directly related to the function, that of an organic cation transporter, identified for the particular protein, hOCTN1, encoded by the claimed nucleic acids. It is <u>substantial</u> because the asserted utility provides a significant currently available real-world benefit: an assay for finding the best cancer treatment for a cell or tissue that expresses the hOCTN1 transporter. The assay can select for the best treatment from among several known carcinostatics that already have

substantial value in treating cancer. **There is no need to know anything more about the physiological role of hOCTN1 in order for the gene to be employed as disclosed.**

This is very different from the facts of the Brenner case. In Brenner, *no* utility was asserted for the compound produced by the claimed process. The only suggested utility in Brenner was proposed after the filing date, and it was based on the structural relationship of the compound to other compounds that *were still being tested* for their anti-tumor properties. The question of whether an asserted utility was specific, substantial or credible did not arise, because none was asserted. Further, the utility that was belatedly suggested after the fact was not "well-established," as the analogous compounds were still being tested for a possible use.

The instant application also presents a very different scenario from the facts of a more recent case that addressed utility, In re Fisher, 421 F.3d 1365, 76 USPQ2d 1225 (Fed. Cir. 2005). In Fisher, the court held that claims drawn to five expressed sequence tags (ESTs) from the maize genome did not have a specific or substantial utility because they were mere research tools to probe underlying genes whose identity and usefulness had yet to be determined. In contrast, the instantly claimed isolated nucleic acids encode a fully sequenced, structurally and functionally characterized protein, hOCTN1. The specification actually demonstrates the utility of hOCTN1 in transporting known carcinostatic compounds. The asserted utility is substantial because it is immediately applicable in a current, real-world sense: to select optimal carcinostatics for uptake by cancer cells/tissues that express the hOCTN1 transporter, as opposed to relying on the discovery of a "potentially" useful compound or gene, as in the Fisher case.

The Examiner alleges, in the Final Office Action and in the subsequent interview of January 10, that in the absence of a physiological role for hOCTN1 and/or an identified nexus between the hOCTN1 protein and a particular disease, isolated nucleic acids encoding hOCTN1 by definition can have no specific or substantial utility. She expresses the further view that, in the absence of such a nexus between the hOCTN1 protein and a particular disease, the compounds screened for transport by hOCTN1 (carcinostatic compounds, in some instances) have no specific or substantial utility. During the interview, the Examiner attempted to support her assertions by saying that there were several recent unpublished decisions by the Board of Patent Appeals and Interferences (BPAI) that stood for this proposition. Because the Examiner was unable to name any of them, Applicant reviewed several recent BPAI decisions that concerned rejections of newly discovered proteins or

nucleic acids for lack of utility. The ones that appeared to Applicant to be most relevant to the present rejection are discussed below. Applicant disagrees with the Examiner's conclusion that the reasoning in these cases dictates a finding of lack of utility in the present case. As explained below, the facts are significantly different in the present case.

The cases that affirm lack of a specific or substantial utility all differ from the instant application because the utilities at issue in those cases were fundamentally *based on* a proposed physiological role, or a proposed role in one or more diseases. Moreover, the proposed physiological and/or disease state roles were speculative at best; they were not specific to the protein, nor substantial, *i.e.*, presently available to provide a real-world benefit.

The instantly asserted utility, on the other hand, is based on the recognized function of hOCTN1 as a transporter of drugs, including carcinostatic compounds, a function that is asserted and taught by the specification and that the Examiner has not challenged. As fully explained and exemplified in the specification, for example, at page 31, lines 5-16, in Examples 6 and 7 and in Figures 9 and 10, this proven transporter function means that the polypeptides can be used in an assay to screen for optimal carcinostatic treatments for cells/tissues expressing hOCTN1, *regardless* of the actual physiological role of hOCTN1 *in vivo*. Further, the transporter function can be used to deliver the carcinostatics identified by the screening assay to any cancer cells and tissues that express the hOCTN1 transporter. That the transporter may also be expressed in normal cells and tissues is irrelevant to its utility as a drug delivery vehicle to target cells and tissues of interest. The delivery of drugs in general does not rely on the availability of a mechanism that is present exclusively in a target of interest. Note, for example, the recognized usefulness of actinomycin D, etoposide, vinblastine and daunomycin in the treatment of cancer, despite the fact that they can be transported into normal cells as well as cancer cells. Thus, the relative expression of hOCTN1 in cancer *vs.* normal cells, and the identity of the cation naturally transported by hOCTN1 into cells, do not have any bearing on hOCTN1's ability to transport carcinostatic compounds identified in a screen as amenable to being transported by hOCTN1.

It may be helpful to analogize the present situation to a hypothetical discovery of a compound extracted from the bark of a tree and disclosed in the specification to be useful as a starting material for synthesis of related compounds having potent anticancer activity against tumors in animals. In this hypothetical fact pattern, nothing is known about the physiological role the compound performs in the tree, nor any link to a known disease of the tree. The

asserted utility is as a starting material for synthesis of anticancer agents, a utility that probably has nothing to do with its physiological role in the tree. There is no question that the compound would be found to have patentable utility despite the lack of information about its physiological role. Similarly, the utility of the presently claimed nucleic acids and their encoded proteins disclosed in the specification does not hinge on knowledge of their physiological role.

Related BPAI Decisions Addressing Utility

The asserted utility of the encoded hOCTN1 polypeptide is based on its ability to be a drug delivery vehicle, which is a function that does not have to be related in any way to its physiological role or its presence or absence in a disease state. This is a very different scenario from the BPAI decisions referred to by the Examiner, where the asserted utilities were based on the physiological role of the encoded proteins and/or their role in a disease state. Some of these decisions are discussed in turn below:

1) In <u>Ex parte Starling and Finger</u> (Appeal No. 2005-2121; decided sometime in 2006 (exact date unavailable)), the claims at issue were directed to isolated nucleic acids encoding a protein designated APEX-1. The APEX-1 protein was identified as belonging to the CD2 subgroup of immunoglobulins, based on its structural features. Applicant in this case argued that the assignment of APEX-1 to the CD2 family in and of itself constituted an asserted utility that is specific, substantial and credible, because the CD2 family "is well-characterized as having utility with respect to leukocyte proliferation, differentiation, migration and activation, and diseases associated therewith." Applicant further argued that, as asserted in the specification, APEX-1 is useful for diagnosing diseases associated with the presence or absence of the APEX protein. The BPAI affirmed the Examiner's decision that the asserted utilities were neither specific nor substantial because (a) the CD2 family members do not share a common function; hence no specific function could be asserted for APEX-1; and (b) Applicant's did not demonstrate a nexus between APEX-1 and a particular disease state or states.

Thus, the asserted utilities in this case were based on properties of APEX-1 that were vaguely defined (not specific, just that it belonged to an immunoglobulin subgroup), and not capable of immediate and significant real-world application (substantial). This is a far cry from the instant case, where the asserted utility is based on a specific, <u>demonstrated</u> function (organic cation transporter) for hOCTN1, and the function is applicable for present and

immediate benefit to the screening of well-known carcinostatics for preferential absorption by hOCTN1. In the present case, the asserted function is not keyed to an unknown physiological role of the protein, but rather to an activity actually demonstrated in experiments (*e.g.*, Examples 6 and 7) disclosed in the specification.

Several other BPAI decisions are based on the same premise as Ex parte Starling, including:

2) Ex parte Turner *et al.*, (Appeal No. 2004-1040; decided September 24, 2004), in which an isolated nucleic acid molecule encoding a protein with "limited structural similarity" to neurexin proteins and contactin-associated proteins, was held to lack specific or substantial utility when the asserted utility at issue was based on the diverse biological functions of neurexins and contactin-associated proteins, their use in identifying potential polymorphisms associated with the new protein (no demonstration that such polymorphisms actually exist), and their use in treating diseases that might be associated with the new protein (no actual diseases established). In this case, the encoded protein additionally had only limited similarity to the neurexins and contactin-associated proteins, thus rendering its identity and possible activity vague and non-specific.

3) Ex parte Donoho *et al.*, (Appeal No. 2004-1103; decided March 28, 2007), in which an isolated nucleic acid encoding a protein *later* (post-filing) demonstrated as having some homology to a G-protein coupled receptor was held to have no specific or substantial utility. In contrast, Applicant has established in the specification both that hOCTN1 is definitely a transporter *and* that it can be utilized in a very specific and useful way.

4) Ex parte Yu and Turner, (Appeal No. 2004-1761; decided sometime in 2004 (date unavailable)), in which an isolated nucleic acid encoding a protein with some similarity to (a) the ADAMTS family of metalloproteinases, (b) receptor-linked phosphatases and (c) membrane-associated cell adhesion proteins, was held to lack specific or substantial utility where the asserted utilities were: processes that identified compounds that modulated this protein with as-yet unknown physiological significance, and, potentially, RFLP analysis if there turned out to be polymorphisms associated with this gene. In contrast, the presently asserted utility has nothing to do with modulating hOCTN1's activity or a mere use to detect polymorphisms that might be discovered in the future. Instead, the asserted utility harnesses an activity that hOCTN1 has been shown to possess.

5) Ex parte Lal *et al.*, (Appeal No. 2007-2517; decided June 29, 2007), in which an isolated polypeptide having some homology to more than one type of G-protein coupled receptor (a taste receptor and a calcium-sensing receptor) was held not to have specific or substantial utility. The post-filing discovery that the protein was in fact a taste-specific receptor was held not to establish utility of the application as filed. In contrast, Applicant has established and disclosed in the specification that hOCTN1 is definitely an organic cation transporter *and* that it can be utilized in a very specific and useful way.

6) Ex parte Lind *et al.*, (Appeal No. 2005-0792; decided May 31, 2005), in which an isolated nucleic acid molecule encoding a G-protein coupled receptor hypothesized to play a role in one or more of a large laundry list of diseases was held to have only insubstantial asserted utilities because the asserted utilities, *all dependent on an as-yet undiscovered nexus between the biological activity of the protein and a disease*, could not be substantiated without a "great deal of further experimentation." In addition, post-filing evidence of homology to a G-protein coupled receptor for asthma susceptibility was held not to satisfy the utility requirement as of the application's filing date. In contrast, the presently asserted utility was fully described and demonstrated in the specification.

7) Ex parte Ramakrishnan, in which the claimed method of screening for therapeutic candidates targeting a dopamine-like G-protein coupled receptor, hypothesized to play a role in one of a large laundry list of diseases, was held not to be a patentable utility because no utility was established for the candidate therapeutic agents so identified. In contrast, the present specification demonstrates that compounds widely recognized as useful anticancer agents are transported by hOCTN1, proving that it is a means for introducing such agents into cells that express hOCTN1 that that the assay will be useful for confirming what drugs can and cannot be transported by hOCTN1-expressing cells.

Clearly, the above cases differ from the instant case in many respects, including:

(1) in some cases, unlike the instant case where hOCTN1 was clearly identified as an organic cation transporter, the newly discovered protein was not definitively identified as belonging to a particular class of proteins with a defined function;

(2) unlike the instant case, where one of the asserted utilities in the specification (screening for the transport of carcinostatics) is applicable regardless of the hOCTN1 polypeptide's physiological role, or its role in a disease, the above cases all asserted utilities

that were based on a physiological role and an involvement in disease for the encoded proteins; and

(3) unlike the instant case, where the encoded protein is presently available to screen for optimal carcinostatic therapies (from among well-known carcinostatic compounds) for uptake in cells/tissues that express hOCTN1, the above cases all asserted utilities that are *merely speculative, pending further research to identify the precise physiological role or disease nexus for each of the encoded proteins.*

Thus, the asserted utility of the isolated nucleic acids in encoding hOCTN1 proteins that transport organic cations, including some that are carcinostatics, is specific and substantiated by experimental evidence (in this case, working examples in the specification). Further, the utility of the encoded hOCTN1 protein in screening for carcinostatics amenable to transport by hOCTN1 is substantial because it is clearly related to the real-world use of hOCTN1 in delivering carcinostatic compounds to target cells and tissues that express hOCTN1. Applicant therefore respectfully submits that the presently claimed nucleic acids have an asserted utility that is specific, substantial and credible.

2) Well-established utility

In addition to having an asserted utility that is specific, substantial and credible, Applicant submits that there is a well-established utility for the instantly claimed subject matter. As the references provided with the attached Appendix show, long before the instant application's earliest priority date, the distinct structural features that characterized organic cation transporter proteins (Maiden *et al.*), and their role in drug uptake and distribution in organs such as intestines (Tsuji), kidneys (Ullrich *et al.*) and the liver (Meijer *et al.*), were well-known. Thus, regardless of what is or is not asserted in the specification, the claimed isolated nucleic acids encode a protein that has a well-established utility, *i.e.*, one of ordinary skill in the art would immediately appreciate why the hOCTN1 protein is useful, based on its structural and functional characteristics as an organic cation transporter. Applicant therefore submits that the utility requirement is met in this case.

III. REJECTION OF CLAIMS 8-11, 13, 16-25, 27, 29-32 AND 36 UNDER 35 U.S.C. §112, FIRST PARAGRAPH - ENABLEMENT

Claims 8-11, 13, 16-25, 27, 29-32 and 36 are rejected under 35 U.S.C. §112, first paragraph, for lack of enablement because the claims allegedly are not supported by a specific and substantial asserted utility, nor a well-established utility. The Examiner further

states that even if the claimed subject matter is eventually deemed to meet the utility requirement, claims 8, 10, 16, 18, 21, 23, 27, 29-31 and 32 nonetheless would remain rejected because the specification allegedly provides little or no guidance, beyond the primary sequence data and consensus sequences, that would permit one of skill in the art to determine positions in the DNA and protein that are tolerant to change, without undue experimentation.

The Examiner concedes that hOCTN2, which is only 76% homologous to hOCTN1, also has organic cation transporter activity. She however states that they are not "variants" of each other because they transport different cations and are therefore different transporters. The Examiner also concedes that Bowie *et al.*, cited by Applicant in the previous response, does discuss how proteins are tolerant of even random substitutions, but then brushes aside its significance by saying that Bowie *et al.* did not examine "examine mutations in receptors or ion transporters." In addition, in discussing the *lac* repressor, Bowie *et al.* allegedly mentions how at some positions, no substitutions or only conservative substitutions were allowed. The Examiner concludes that a "large quantity of experimentation" is necessary to generate the "infinite number of variants" recited in the claims, and they are therefore not enabled for their full scope.

Applicant traverses this rejection. To the extent the enablement rejection is a corollary to the rejection on grounds of lack of utility, the above arguments addressing utility establish that the specification at the time of filing clearly taught how to make and use the claimed isolated nucleic acids in assays where the encoded hOCTN1 protein is expressed and screened for its ability to transport a variety of organic cations, including carcinostatic compounds. The transport activity is demonstrated in several working examples (Examples 6-8, for instance).

With regard to variants of hOCTN1, Applicant maintains that, given the detailed teachings of the specification regarding the structural motifs that confer organic cation transporter activity on a protein, and the identification of these structural motifs in the hOCTN1 organic cation transporter, one can modify the hOCTN1 protein at a high percentage of its amino acid residues in a manner that retains the characteristics of an organic cation transporter. This is borne out by the three specific examples of organic cation transporters in the specification, other than hOCTN1, which bear a 50% -76% homology to the hOCTN1 organic cation transporter.

The Examiner alleges that the transporter that is 76% homologous to hOCTN1, hOCTN2, is a different transporter and not merely a "variant" of hOCTN1. Applicant notes that the subject claims do not use the term "variant of hOCTN1." The specified function is "a transporter of an organic cation," a function that is present in each of the disclosed polypeptides. Moreover, the mouse OCTN1 peptide is only 50% homologous to hOCTN1, yet belongs to the same family as hOCTN1 and functions as an organic cation transporter.

Notwithstanding the above, in the interest of placing the application in condition for allowance or reducing the number of issues for appeal, claim 8 is amended to remove reference to 70% sequence identity, and claims 17, 22, and 29-31 are canceled. Claim 10, which specifies that the claimed nucleic acid encode a polypeptide having between one and thirty <u>conservative</u> amino acid substitutions in the hOCTN1 sequence, is retained (in the 551 amino acid sequence, this represents variants whose sequences are between 94.6% to 99.8% identical to the hOCTN1 sequence, with all substitutions being conservative ones). Also retained is claim 32, directed to nucleic acids that encode proteins containing even fewer substitutions (i.e., one to ten, corresponding to identity of 98.1-99.8%, with all substitutions being conservative ones). As the Examiner pointed out during the interview of January 10, methods of generating variants of a protein that are 95% or more identical to the protein and retain the activity of the protein are generally accepted as enabled. In this instance, a variant with up to thirty conservative amino acid substitutions in hOCTN1 could have a sequence identity that is slightly lower, *i.e.*, 94.6%. However, the claim language specifies that the amino acid substitutions are conservative and not random. Applicant, therefore, respectfully submits that claims 10, 18, 23 and 32 are enabled for their full scope.

IV. THE REJECTION OF CLAIMS 8, 10, 16, 18, 21, 23, 27, 29-31 AND 32 UNDER 35 U.S.C. §112, FIRST PARAGRAPH – WRITTEN DESCRIPTION

Claims 8, 10, 16, 18, 21, 23, 27, 29-31 and 32 are rejected under 35 U.S.C. §112, first paragraph, as lacking adequate written description. The Examiner alleges that the specification adequately describes only the nucleic acid having the sequence set forth in SEQ ID NO:2 and the polypeptide having the sequence set forth in SEQ ID NO:1 (hOCTN1). She further alleges that there is inadequate written description for variants that are 70%, 80% or 90% identical to SEQ ID NO:1, yet retain the activity of hOCTN1, because the art-accepted convention is that it is not possible to modify more than 5% of the amino acid residues of a protein without affecting its activity. She again reiterates that the hOCTN2 protein that

shares 76% homology with hOCTN1 is a completely different transporter, and not merely a variant.

Applicant disagrees with the Examiner. The variant sequences described in the specification (50%, 54% and 76% homologous to hOCTN1) clearly have organic cation transporter activity, thus evidencing possession of functional organic cation transporters that share far less than 95% sequence identity with hOCTN1. Furthermore, Applicant is unaware of any basis for the assumption that altering more than 5% of the sequence of a protein will necessarily alter its function. Nonetheless, in the interest of placing the application in condition for allowance or reducing the number of issues for appeal, claim 8 is amended to remove reference to a sequence variant that is 70% identical to SEQ ID NO:1, and claims 17, 22 and 29-31 are canceled. Claims 10 (and its dependents) and 32, which encompass nucleic acids encoding polypeptides that contain between one and thirty or between one and ten conservative amino acid substitutions in the hOCTN1 sequence, are retained. (As noted above, in the 551 amino acid sequence, this represents polypeptides whose sequences are respectively 94.6 to 99.8% or 98.1 to 99.8% identical to the hOCTN1 sequence).

* * *

In view of the above, reconsideration and allowance of the application are respectfully requested.

Respectfully submitted,

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APPENDIX

(1) Maiden *et al.*, *Nature*, 325:641-643 (1987)

(2) Meijer *et al.*, *J. Pharmacokin. Biopharm.*, 18:35-70 (1990)

(3) Ullrich *et al.*, *Clin. Investig.*, 71:843-848 (1993)

(4) Tsuji et al., *Pharm. Res.*, 13(7):963-1132 (1996)

Mammalian and bacterial sugar transport proteins are homologous

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The uptake of a sugar across the boundary membrane is a primary event in the nutrition of most cells, but the hydrophobic nature of the transport proteins involved makes them difficult to characterize. Their amino-acid sequences can, however, be determined by cloning and sequencing the corresponding gene (or complementary DNA). We have determined the sequences of the arabinose-H^+ and xylose-H^+ membrane transport proteins of *Escherichia coli*. They are homologous with each other and, unexpectedly, with the glucose transporters of human hepatoma[1] and rat brain[2] cells. All four proteins share similarities with the *E. coli* citrate transporter[18]. Comparisons of their sequences and hydropathic profiles yield insights into their structure, functionally important residues and possible evolutionary relationships. There is little apparent homology with the lactose-H^+ (LacY)[3] or melibiose-Na^+ (MelB)[4] transport proteins of *E. coli*.

In the bacterium *Escherichia coli* there is one H^+-linked transport system for arabinose encoded by the gene *araE*[5], and another for xylose encoded by *xylE*[6] (with gene products AraE and XylE). The linkage of sugar and H^+ translocation enables energization of nutrient uptake by the transmembrane electrochemical proton gradient[7,8].

We have isolated and sequenced the *araE* and *xylE* genes, and deduced the amino-acid sequence of each protein (Fig. 1). AraE contains 472 amino acids (relative molecular mass (M_r)51,683), and XylE contains 491 amino acids (M_r 53,607). Both are typical membrane proteins: they are very hydrophobic (about 67% nonpolar residues); their hydrophobic and hydrophilic regions are arranged alternately (Fig. 1; see also ref. 9); and their calculated M_r values are substantially higher than the values of ~36,000 for AraE[8] and 39,000 for XylE[8] measured by SDS-polyacrylamide gel electrophoresis.

The amino-acid sequence of the glucose transport protein, which does not translocate a cation, from a human hepatoma cell line (HepG2) was recently determined[1]. The reading frame

```
X ------------MNTQYNSSYIFSITLVATLGGLL-FGYDTAVISGTVESLNTVFVAPQ-  46
G ---------MEPSSKKLTGRL-M-LAVGGAVLGSLQFGYNTGVINAPQKVIEE-FYNQTW  48
A MVTINTESALTPRSLRDTRRMNMFVSVAAAVAGLL-FGLDIGVIAGALPFITDHFVLTS-  58

C -----MTQQPSRAGTFGAILRVTSGNFLEQFDFFL-FGFYATYIAKTFFPAESEFAALM-  53

X --NLSESAANSLL----GFCVASALIGCIIGGALGGYCSNRFGRRDSLKIAAVLFFISGVG 101
G VHRYGESILPTTLTTLWSLSVAIFSVGGMIGSFSVGLFVNRFGRRNSMLMMNLLAFVSAVL 109
A --RLQE------------WVVSSMMLGAAIGALFNGWLSFRLGRKYSLMAIAILFVLGSIG 105

C ---------------LTFAVFGSGFLMRPIGAVVLGAYIDRIGRRKGLMITLAIMCCGTLL  99

X SAWPELGFTSINPDNTVPVYLAGYVPEFVIY-RIIGGIGVGLASMLSPMYIAELAPAHIRG 161
G MGFSKLGK----------------SFEMLILGRFIIGVYCGLTTGFVPMYVGEVSPTAFRG 154
A SAFAT-------------------SVEMLIAARVVLGIAVGIASYTAPLYLSEMASENVRG 147

C IALVPGYQTIGLLAPVLVL-----------VGRLLQGFSAGVELGGVSVYLSEIATPGNKG 149

X KLVSFNQFAIIFGQLLVYCVNYFIARSGDASWLNTDGWRYMFASECIPALLFLMLLYTVPE 222
G ALGTLHQLGIVVGILIA------QVFGLDSIMGNKDLWPLLLSIIFIPALLQCIVLPFCPE 209
A KMISMYQLMVTLGIVLA--------FLSDTAFSYSGNWRAMLGVLALPAVLLIILVVFLPN 200

C PYTSWQSASQQVAYVVAALIGYGLNVTLGHDEISEWGWRIPFFIGCMIIPLIFVLRRSLQE 210

X SPRWLMS-RGKQEQAEGILRKIMGNTLAT-QAVQEIKHSLDHGRKTGG-------RLLMFG 274
G SPRFLLINRNEENRAKSVLKKLRGTADVTH-DLQEMKEESRQMMREKKVTILELFRSPAY- 268
A SPRWLAS-KGRHIEAEEVLRMLRDTSEKAREELNEIRESL--KLKQGGWA---LFKINRNV 255

C TEAFL---QRKHRPDTREIPTTIA----------------------------------KNW 234

X VGVIVIGVMLSIFQQFVGINVVLYYAPEVFKTLGAS-TDIALLQ-TIIVGVINLTFTVLAI 333
G RQPILIAVVLQLSQQLSGINAVFYYSTSIFEKAGV----QQPVYATIGSGIVNTAFTVVSL 325
A RRAVFLGMLLQAMQQFTGMNIIMYYAPRIFKMAGFTTTSQQMI-ATLVVGLTFMFATFIAV 315

C R-IITAGTLLVAMTT-TTFYFITVYTPTYGRTVLNL-SARDSLVVTMLVGISNFIWLPIGG 292

X MTVDKFGRKPLQIIGALGMAIGMFSLGTAFYT---QAP-----GIVALLSMLFYVAAFAMS 386
G FVVERAGRRTLHLIGLAGMA-GCAILMTIALALLEQLPWM---SYLSIVAIFGFVAFFEVG 382
A FTVDKACGKPALKIGFSVMALGTLVLGYC-LMQFDNGTASSGLSWLSVGMTMMCIAGYAMS 375

C AISDRIGRRPVLMGITLLALVTTLPVMNWLTAAPD---------FTRMTLVLLWFSPFPGM 344

X WGPVCWVLLSEIFPNAIRGKALAIAVA--AQWLANYFVSWTFPMMDKNSWLVAHFHNGFSY 445
G PGPIPWFIVAELFSQGPRP--AAIAVAGFSNWTSNFIVGMCFQY-------VEQLCGPYVF 434
A AAPVVWILCSEIQP--LKCRDFGITCSTTTMWVSMMIIGATFLTL------LDSIGAAGTF 428

C YMGAMVAALTEVMPVYYVRTVGPSLAPSLATAIFGCLTPAISTALVQ-----LTCDKSSPGW 400

X WIYG-CMGVLAALFMWKFVPETKGKTLEELEALWEPETKKTQQTATL------------ 491
G IIFT-VLLVLFFIFTYFKVPETKGRTFDEIASGFRQGGASQSDKTPEELFHPLGADSQV 492
A WLYT-ALFIAFVGITFWLIPETKNVTLEHIERKLMAGEKLRNIGV-------------- 472

C VLMCAALCGLAATTMLFARLSSGYQTVEMKL---------------------------- 431
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Fig. 1 Aligned sequences of the *E. coli* xylose-H^+, arabinose-H^+ and citrate transporters with the human hepatoma glucose transporter. The sequences of the glucose and citrate transport proteins were taken from refs 1 and 18, respectively. Residues are boxed in the XylE, glucose and AraE transporters if they occur more than once at an aligned position in the three sugar transporter sequences. They are only boxed in the citrate transporter if they also occur in the aligned positions of two or three of the sugar transporters. The predicted membrane-spanning regions of the glucose transporter[1] are underlined. X, XylE; A, AraE; C, citrate transporter; G, glucose transporter.

Methods. The *araE* gene is located at 61 min on the linkage map close to the *lysA* gene[8]. A restriction map of a λ*plac* Mu phage inserted into the *araE* gene established its precise position by comparison with an overlapping restriction map of the *lysA* region[36]. This enabled a 2.8-kilobase (kb) DNA fragment containing the intact *araE* gene and promoter region to be cloned from a specialized λ transducing phage[37] into the multicopy vector pBR322. The resultant plasmid, pMM25, conferred arabinose-H^+ transport activity on appropriate recipient *E. coli* strains negative for *araE*. The *xylE* gene is located at 91.4 min on the linkage map between the *pgi* and *malB* genes[6]. A restriction map of a λ*plac* Mu phage inserted into the *xylE* gene established its precise position by comparison with an overlapping restriction map of the *malB* region[38]. This enabled a 2.8-kb DNA fragment containing the intact *xylE* gene to be cloned from a λ (*xylE*) Φ (*malK'-'lacZ*) phage into the multicopy vector pBR328. The resultant plasmid, pEJ3, conferred xylose-H^+ transport activity on appropriate recipient *E. coli* strains deleted for *xylE*. The complete DNA sequence of each 2.8-kb fragment and criteria for identifying the reading frame will be described elsewhere.

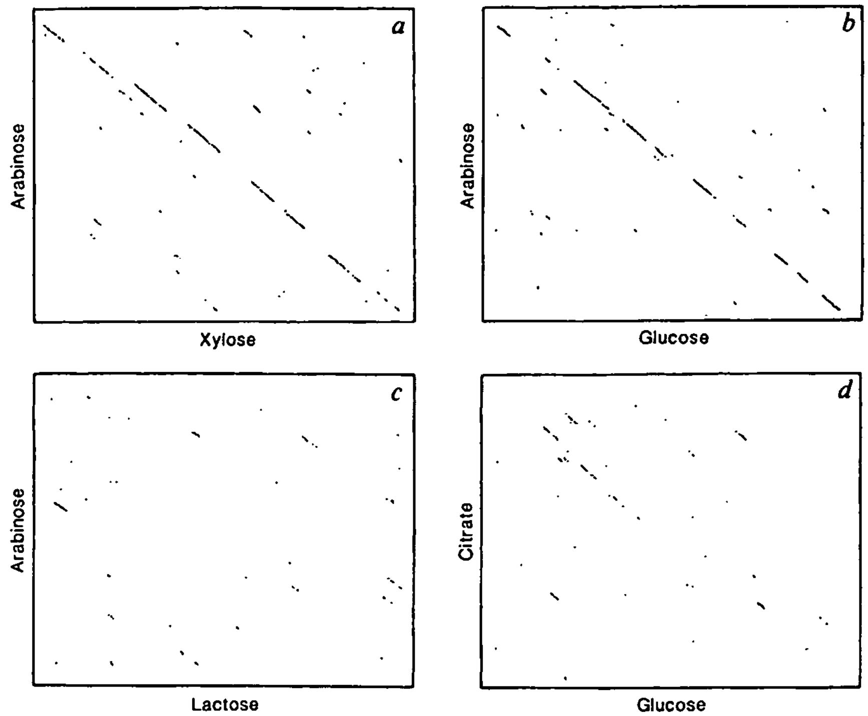

Fig. 2 Comparisons of the sequences of transport proteins using Diagon plots[10]. *a*, The arabinose-H^+ and xylose-H^+ transport proteins of *E. coli*; *b*, the arabinose-H^+ transport protein of *E. coli* and glucose transport protein of human hepatoma cells; *c*, the arabinose-H^+ and lactose-H^+ transport proteins of *E. coli*; *d*, the citrate transport protein of *E. coli* and the glucose transport protein of human hepatoma cells. The comparisons used a 21-residue segment and generated a dot if the score exceeded 240 (ref. 10).

was confirmed by the identity of parts of its sequence with peptides derived from the erythrocyte glucose transporter[1]. Its amino-acid composition was similar to that of the AraE and XylE proteins with 492 amino acids (M_r 54,117)[1]. The sequence of the glucose transporter from rat brain cells, identified and cloned using similar techniques, was virtually identical, having 492 amino acids (M_r 56,133)[2].

The sugar-transporter sequences were compared using the Diagon algorithm of Staden[10]. Several homologous regions were revealed (Fig. 2*a*, *b*) in both hydrophobic and hydrophilic regions (Fig. 1). Furthermore, their hydropathic profiles[9,11] were similar (Fig. 3); there was an hydrophilic segment, 50-65 residues long, located approximately at the mid-points of each of the sequences (Fig. 3), with some similar hydrophilic segments either side marked X in Fig. 3, and a similar number (12) of hydrophobic segments (Fig. 3).

Diagon plots drawn at the same and less stringent levels of discrimination did not reveal such homologies between XylE, AraE or the glucose transporter and the lactose[3] or melibiose[4] transporters of *E. coli* (one example is shown in Fig. 2*c*). It should be noted that the scoring matrix used by the Diagon algorithm was not devised for membrane proteins.

The sequence of the passive glucose transporter[1] is aligned with the sequences of the active arabinose and xylose transport proteins in Fig. 1. Seventy-seven residues are conserved in all three proteins (the glucose transporter has 131 identitites with AraE and 142 identities with XylE; AraE and XylE have 141 residues in common). There are additional conservative substitutions[12] throughout the sequences. Consequently nearly 40% of residues can be regarded as homologous, a level sufficient to expect similar secondary and tertiary structures for all three proteins. Like many integral membrane proteins[13] these transporters lack the N-terminal signal sequences typically required for insertion of proteins through membranes[14]. The attachment site for the carbohydrate chain, Asn 45 of the hepatoma cell glucose transporter[1,15], is not conserved in the XylE or AraE proteins (Fig. 1).

The citrate-H^+ transporter[16] of *E. coli* has 431 amino acids (M_r 46,979)[17,18] and an apparent M_r of 35,000[19] (determined by SDS-polyacrylamide gel electrophoresis. Diagon comparisons revealed little homology with AraE or XylE, but some homology with the glucose transporter (Fig. 2*d*). However, all four proteins had similar hydropathic profiles (Fig. 3) with 12 membrane-spanning regions predicted by the Eisenberg algorithm[11] (Fig. 1, Fig. 3). This enabled us to align the patterns of conserved amino acids in all four transporters, particularly in the regions 30-49, 60-101, 121-149, 257-302 (Fig. 1). These patterns may be coincidental[20], but, more probably, they indicate that those residues boxed in the citrate transporter (Fig. 1) are critical for the common transport function of all the proteins.

It is interesting to locate certain conserved amino acids, in view of previous suggestions as to their roles in transport. For example, a glutamate (or aspartate) residue is responsible for H^+-translocation by subunit c in the F_0 moiety of H^+-ATPase[21], and Glu 325 is implicated in H^+-translocation by LacY[22], so the conservation of such residues at positions corresponding to 153, 337, 397 and 472 of XylE (Fig. 1) identifies putative H^+-translocating residues in these proteins. The mammalian transporters are not thought to translocate cations, however[23].

There is no conservation of histidines and cysteines (Fig. 1), residues implicated in the transport function of LacY[24-27]. Hence they are unlikely to have a common role in these proteins. In all three sugar transporters substrate(s) protect a thiol group against reaction with *N*-ethylmaleimide[8,28,29], a phenomenon well established in LacY[23,24,30]. As all cysteines in AraE occur between residues 343-400, at least a part of this region of this protein is adjacent to a substrate binding site.

A compelling conserved feature is the motif RXGRR, which is duplicated in each of the four proteins (Fig. 1; Fig. 3 marked X; R may be replaced by K), and occurs in a similar form in LacY and MelB (Fig. 3). It is predicted by the Robson algorithm[31] to form a β-bend in six of its eight locations. This may link two helices in a defined topology stabilized by charge-charge interactions with head groups of the lipids. Its sequence also resembles peptides recognized by mammalian cyclic AMP protein kinase[32,33].

The central hydrophilic regions of all four proteins are predicted by the Robson algorithm[31] to be α-helices, which correlates with the observation that 20% of the homologous erythrocyte glucose transporter forms non-membrane α-helix[34]. There are

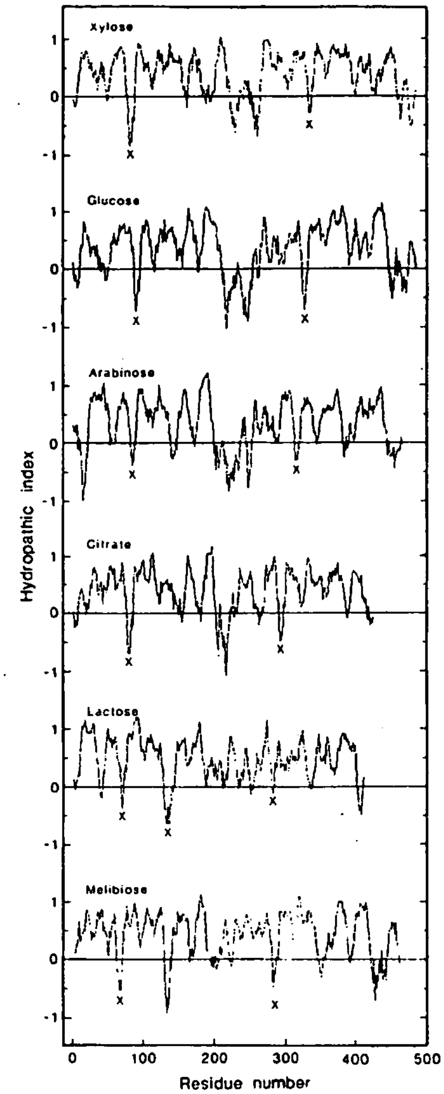

Fig. 3 Hydropathic profiles of transport proteins. Hydropathy values[9] for a window of nine amino-acid residues were averaged and plotted according to the position of the middle residue along the length of each sequence. The sequences of the indicated proteins were taken from Fig. 1 and refs 1, 18, 3 and 4. X indicates the positions of the −RXGRR− motif in each protein.

indications, such as the duplicated RXGRR motif, that the N-terminal and C-terminal halves are arranged symmetrically about this region, with six corresponding membrane α-helices on each side. An internal gene duplication event[20] is thus implied in an ancestral transporter, which then evolved to the present proteins.

The similarity between the sugar transporters from such divergent organisms as bacteria and mammals is probably too great to have arisen by convergent evolution. Gene transfer from eukaryotes to their prokaryotic symbionts remains an explanation: an example of this process has recently been described[35]. But we think it more likely that the homologies reflect functionally important parts of an ancient sugar transporter present in organisms before their divergence into prokaryotes and eukaryotes. If this is the case, homologous sugar transporters may also be found in plants, unicellular eukaryotes and other organisms. Whatever its origin, the similarity of these transport proteins suggests that a combination of biochemical, immunological and genetic techniques can now be used to exploit the advantages of working with prokaryotes to illuminate transport processes in higher organisms.

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Molecular dynamics studied by analysis of the X-ray diffuse scattering from lysozyme crystals

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It is now well established that the biological activity of proteins is related not only to their mean molecular structure, but also to their intramolecular mobility[1–4]. Nearly all techniques sensitive to dynamics have given evidence for intramolecular mobility in proteins: NMR[5,6], ESR[7], Raman spectroscopy[8,9], fluorescence quenching[10], Mössbauer spectroscopy[11], neutron scattering[12], measurements of elastic constants[13] and hydrogen–deuterium exchange[14]. The dynamics of proteins has also been approached by theoretical calculations[15,16]. We report here investigations of the atomic and molecular displacements in hen egg-white lysozyme crystals using a new technique. This technique, based on the X-ray diffuse scattering analysis (scattering out of the Bragg reflections),

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Carrier-Mediated Transport in the Hepatic Distribution and Elimination of Drugs, with Special Reference to the Category of Organic Cations

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Carrier-mediated transport of drugs occurs in various tissues in the body and may largely affect the rate of distribution and elimination. Saturable translocation mechanisms allowing competitive interactions have been identified in the kidneys (tubular secretion), mucosal cells in the gut (intestinal absorption and secretion), choroid plexus (removal of drug from the cerebrospinal fluid), and liver (hepatobiliary excretion). Drugs with quaternary and tertiary amine groups represent the large category of organic cations that can be transported via such mechanisms. The hepatic and to a lesser extent the intestinal cation carrier systems preferentially recognize relatively large molecular weight amphipathic compounds. In the case of multivalent cationic drugs, efficient transport only occurs if large hydrophobic ring structures provide a sufficient lipophilicity-hydrophilicity balance within the drug molecule. At least two separate carrier systems for hepatic uptake of organic cations have been identified through kinetic and photoaffinity labeling studies. In addition absorptive endocytosis may play a role that along with proton-antiport systems and membrane potential driven transport may lead to intracellular sequestration in lysosomes and mitochondria. Concentration gradients of inorganic ions may represent the driving forces for hepatic uptake and biliary excretion of drugs. Recent studies that aim to the identification of potential membrane carrier proteins indicate multiple carriers for organic anions, cations, and uncharged compounds with molecular weights around 50,000 Da. They may represent a family of closely related proteins exhibiting overlapping substrate specificity or, alternatively, an aspecific transport system that mediates translocation of various forms of drugs coupled with inorganic ions. Consequently, extensive pharmacokinetic interactions can be anticipated at the level of uptake and secretion of drugs regardless of their charge.

KEY WORDS: carrier-mediated transport; organic cations; cationic drugs; hepatobiliary elimination; hepatic distribution; drug interactions; structure-pharmacokinetic relationship; multiplicity in carrier proteins.

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INTRODUCTION

The liver performs a variety of functions to maintain whole body homeostasis, such as uptake and processing of nutrients absorbed from the intestine, cholesterol metabolism and bile formation, metabolic conversion of exogenous and endogenous substances by the phase I cytochrome P450 system and by the phase II synthetic conjugation reactions, and many others. Hepatic function is essential for disposition of many drugs and toxic agents (see Fig. 1 for the liver structure). Compounds can be taken up from the bloodstream into the liver and subsequently metabolized, excreted in bile, or transported back to the blood. Relatively lipophilic drugs enter the hepatic parenchymal cells by passive diffusion in their undissociated form (1,2). For more hydrophilic compounds, for instance, glycosides and agents with a negative or positive charge at physiological pH, specialized uptake processes such as carrier-mediated transport are generally necessary for penetration into the cells.

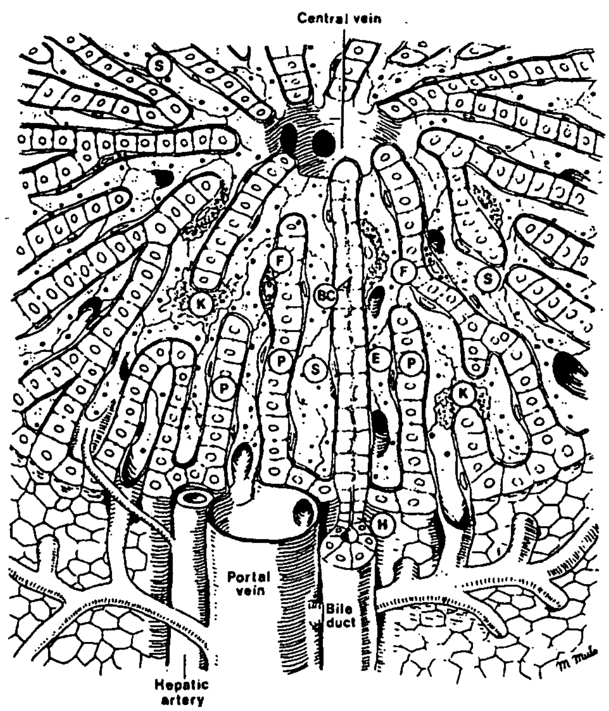


Fig. 1. Schematic figure of the three dimensional structure of hepatic tissue. BC, bile canaliculus; H: Hering canal; E: endothelial cell; F: fat-storing cell; P: parenchymal cell; K: Kupffer cell; S: fenestrated sinusoid. [Adapted from M. Muto (178).]

A large category of drugs consists of compounds with one or more quaternary or tertiary amine functions. Quaternary ammonium groups with a nitrogen linked to four carbon atoms are permanently positively charged (Fig. 2). The tertiary amines potentially are also cations since, at a pK_a of the basic group of ≥ 7.5, at physiological pH the major part of the molecules is protonated. Based on their charge the organic cations are generally divided into monovalent and bivalent cations. Among the monoquaternary drugs are anticholinergic, antineoplastic, and antihelmintic agents. The bisquaternary compounds represent predominantly neuromuscular blocking agents. The tertiary amines include a wide variety of drugs, including local anaesthetics, psychotropic amines, sympathomimetic and sympatholytic agents as well as antihistaminics.

Cationic drugs can pass membranes by carrier-mediated transport and such processes have been characterized in the kidney tubular cells (3–5) explaining secretion of basic drugs from blood into the urine, in intestine as related to secretion of organic cations from blood into the intestinal lumen (6), in the choroid plexus as a mechanism to remove cationic compounds from the liquor to blood (5) as well as in the liver mediating hepatobiliary excretion of cationic drugs (1,5,7,8).

Structure–kinetic relationship has been studied with series of monovalent (9–12) and bivalent organic cations (8,13,14). In all of the excretory organs a two-step carrier-mediated transport seems to be involved: uptake into the cells and excretion from the cells. At both levels saturation

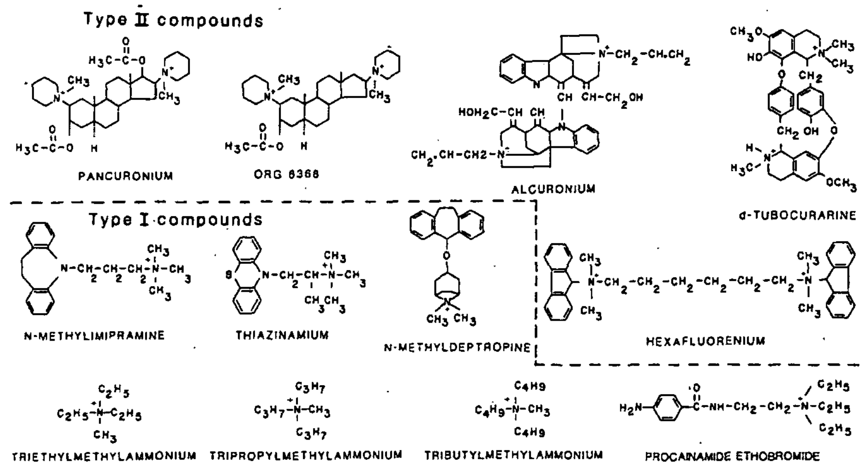


Fig. 2. Chemical structures of organic cationic model compounds. Ethyl-procainamide and tributylmethylammonium represent Type I compounds of relatively low molecular weight. d-Tubocurarine and pancuronium represent Type II compounds being relatively large molecular weight compounds with the cationic centers enclosed in or close to bulky ring structures.

and competitive phenomena have been described (15–22). The hepatic transport of monovalent organic cations, often with a spatial separation of the cationic group from the aromatic ring structure (Type I compounds, see Fig. 2), has traditionally been characterized using the model compound procainamideethobromide, PAEB) or its ^{4}N-acetylated derivative (5,15–21) but also, more recently, with some aliphatic methyl-ammonium compounds (9,10,28). Endogeneous substrates for this transport system may include choline and thiamine (23,24) as well as nicotinamide riboside (25). Transport of organic cations was shown to be sensitive to temperature changes, anoxia, and metabolic inhibitors (16,22,26,27). Multivalent organic cations only seem to undergo significant hepatobiliary transport if the presence of the cationic groups is masked by bulky ring structures (1,8,14) and uptake occurs via a process that differs from that of the Type I compounds (17). They represent the Type II class of organic cations (Fig. 2). At the canalicular pole of the cell the bulky organic cations and the PAEB-like agents may share one carrier-mediated secretion process as evidenced by mutual interactions at that level (17,28). In some cases net canalicular transport can be promoted by bile salts through choleretic effects, ion pair formation or binding to biliary micelles (1,29) but this is certainly not a general rule, especially if uptake into the cells is rate limiting in the hepatobiliary transport (30).

STRUCTURE-PHARMACOKINETIC RELATIONSHIP OF MONOVALENT ORGANIC CATIONS

Several reviews have been published on the hepatic (8,27), renal (4,31), and intestinal (6,32) transport of organic cations. In general, quantitatively important hepatobiliary elimination is restricted to drugs with a relatively large molecular weight (33). A so-called "molecular weight threshold" was proposed above which the biliary excretion of drugs becomes appreciable ($>10\%$ of the dose). For monovalent organic cations the molecular weight threshold was suggested to be 200 ± 50 Da in the rat (12). However, molecular weight indirectly reflects other physicochemical characteristics, such as lipophilicity. In this context Schanker (5) noted that the chemical structures of organic cations that are secreted into bile have the following characteristics in common: a positively charged quaternary ammonium group at one end of the molecule and one or more nonpolar ring structures at the opposite end. Several later studies (10,11,34) indicated that the lipophilicity of the organic cations appears to be a better parameter for the prediction of hepatobiliary transport. However it should be noted that the above-mentioned data are related to transport from blood to bile, not allowing a discrimination between the hepatic uptake and the biliary

excretion processes. Data from Neef *et al.* (9) showed that even hydrophilic, low molecular weight cations (< 200 Da) are substantially accumulated in the liver within 10 min; no clear relation with lipophilicity was observed with regard to hepatic uptake. In contrast, the biliary excretion of the monovalent cations increased with increasing lipophilicity (9,10), thus suggesting that lipophilicity is predominantly a prerequisite for efficient transport from the hepatocyte into the bile. Yet the extent of lipophilicity alone cannot fully explain hepatobiliary transport profile of organic cations, as was shown in a study with thiazinamium and its very polar sulfoxide metabolite, which are equally well excreted in bile (35). This suggests that not the lipophilicity *per se* but rather the balance between hydrophilic and hydrophobic properties plays a crucial role. Even the tricyclic ring structure including the polar sulfoxide group may provide sufficient opportunity to interact with the supposed carrier sites. With the presence of the positively charged group, the presence of a planar ring structure some distance from the cationic moiety may represent a minimal condition for drug-carrier interaction. In addition, the differences in the hepatobiliary transport of two stereoisomers of oxyphenonium indicate that the spatial structure may also influence transport (36).

A study with 14 organic cations (Fig. 3) showed that while biliary excretion becomes more important with increasing lipophilicity, no evident relation with lipophilicity exists for renal clearance (10). These observations are in line with the idea of Rennick (31) that only the positive charge of the molecule is important for tubular secretion. Quaternary amines are transported more efficiently than the tertiary ones which in turn are transported more effectively than the secondary ones (4). Yet recent data indicate that for organic cations and anions several subsystems with overlapping substrate specificity exist in the kidney (3). This implies that more specific molecular features may play a role in renal excretion. This is also indicated by the differences in the tubular secretion of several stereoisomers of organic cations (3) and by the different transport of MPP^+ and MPTP in renal brush border membrane vesicles (37).

With respect to the secretory function of the intestine, studies from the group of Lauterbach (38) demonstrated that the permeation of quaternary ammonium compounds across jejunal mucosa proceeds much faster from the blood side into the luminal compartment than in the absorptive direction, indicating intestinal secretion (38,39). The secretion of organic cations occurs by a saturable mechanism and can be inhibited by structurally related compounds (32). The luminal system, secreting the organic cations against the membrane potential, was inhibited by anaerobiosis, whereas the uptake at the basolateral membrane was not affected (38). Similar to hepatic elimination, intestinal secretion of organic cations increases with increasing

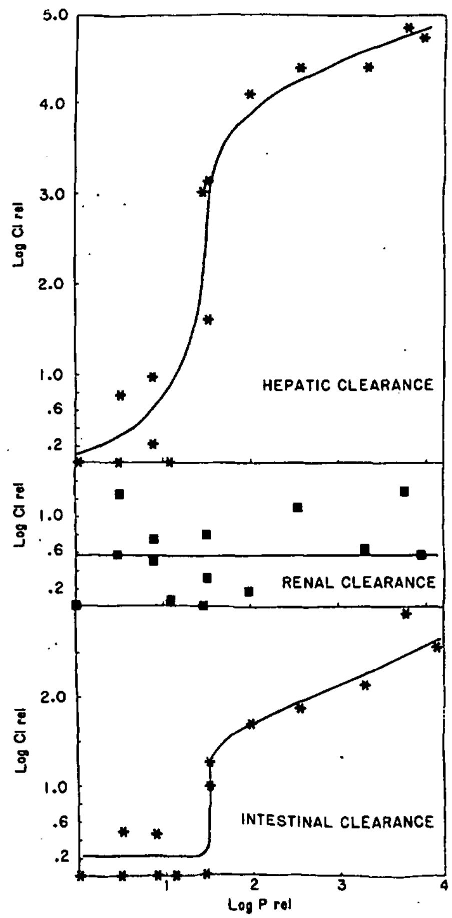


Fig. 3. Relation between lipophilicity (measured by partition between octanol-Krebs buffer and expressed relative to the value of tetraethylammonium) and carrier-mediated clearance of 14 monovalent organic cations (drugs with a quaternary ammonium group) via biliary, intestinal, and renal routes in the rat. The clearance values depicted are corrected for passive fluxes (glomerular filtration etc.), blood flow limitation, and protein binding and indicate carrier-mediated transport in the organs. Between log $P = 1$ and log $P = 2$, biliary clearance increases about 1000 and intestinal clearance about 100 times in contrast to renal clearance that shows no such correlative pattern.

lipophilicity of the compound (10,38). In accordance with the phylogenetic origin of the liver from a gut diverticulum, the secretory system in the intestine may be more closely related to that in the liver than to that in the kidney (6).

STRUCTURE–PHARMACOKINETIC RELATIONSHIP OF BIVALENT ORGANIC CATIONS

In general, nondepolarizing neuromuscular blocking agents are characterized by two cationic centers in the molecule. The elucidation of their kinetics is of major importance to achieve muscle relaxation of predictable intensity and duration of action (40–42). Among the major determinants in the pharmacokinetics, and thus the course of the muscle-relaxing effect, are the distribution and the elimination of the muscle relaxant. Due to their high hydrophilicity, the distribution volume of the muscle relaxants is approximately limited to that of the extracellular fluids (43). Yet some association with specific tissue components occurs. Muscle relaxants have a great affinity for the acid mucopolysaccharides including chondroitin sulfuric acids of the connective tissues, where high tissue concentrations for various muscle relaxants have been found (44–48).

Biotransformation, renal and hepatic excretion are the main processes contributing to the elimination of muscle relaxants from the body. In man, biotransformation of nondepolarizing muscle relaxants, in general, exerts little influence on the elimination (43). Many relaxants are excreted unchanged and if metabolities are formed, they account for only small proportions of the administered dose (49–51). An exception in this respect is atracurium, which is rapidly degraded to inactive breakdown products partly by Hoffman elimination and by direct reactions with nucleophiles (glutathione, cysteine) in plasma (52,53). These nonbiological methods of degradation occur at physiological temperature and *p*H (52,53). In addition, enzymatic ester hydrolysis contributes to the degradation of atracurium (53).

In general, urinary excretion forms the major elimination pathway for these muscle relaxants. This holds especially for gallamine (54), alloferine (55), metocurine (56), pancuronium (57), pipecurium (58,59), and chandonium (60) and is generally accompanied with a relatively long duration of action. Hepatic elimination presents an alternative route of elimination for many relaxants. If significant biliary excretion occurs for a given agent, often distribution to the liver is rapid and this process of rapid removal from the circulation alone may terminate the action of the compound (61). Vecuronium is predominantly excreted in bile (61) and its efficient hepatic uptake from the circulation is a major determinant of its short duration of

action (61,62). (Fig. 4). Hexafluronium is excreted mainly in bile in rat and in man (63), while the biliary route is also an important way of elimination for d-tubocurarine (28,56). Rapid uptake of stercuronium in the liver is the major factor determining the short duration of action in the rat (44). In the cat the liver was shown to accumulate substantial amounts of pancuronium and its congeners dacuronium and Org 6368 (49). It is obvious that impaired liver or kidney function, often, more or less, affects the elimination rate of the relaxants. The clinical implications of this impaired organ function with respect to the elimination of curare-like agents have recently been reviewed by Booij (64).

With respect to the molecular features determining the route of elimination, a molecular weight threshold of 500-600 Da has been proposed for hepatobiliary excretion of bivalent organic cations (14). Similar to the monovalent cations, lipophilicity seems to be an important factor in determining the elimination pathway of bivalent organic cations (13,65,66). The importance of the lipophilicity is clearly reflected in the different hepatic disposition of d-tubocurarine and its less lipid soluble trimethyl derivative metocurine (66) as well as in the very dissimilar kinetics of vecuronium and pancuronium (67,68), which differ in only one methyl group at one of

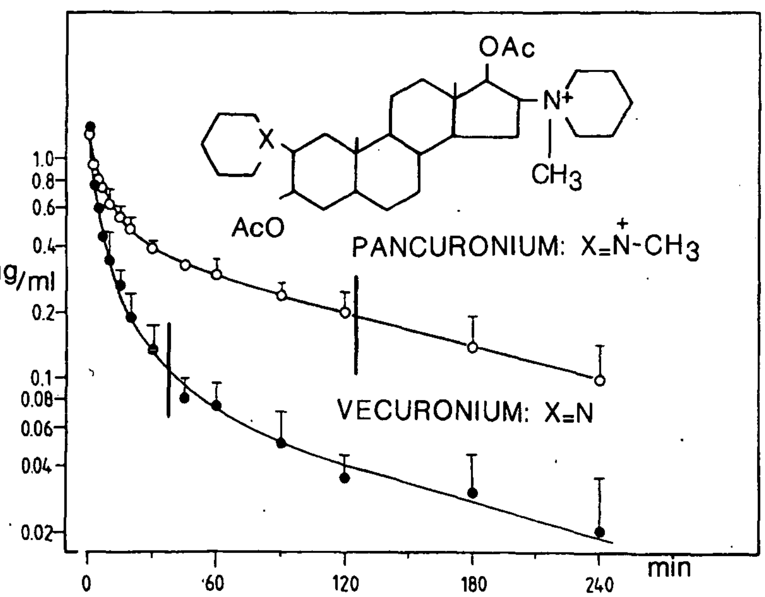
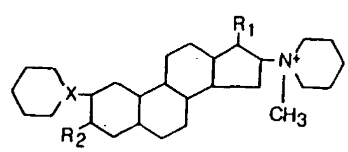


Fig. 4. Plasma concentrations of vecuronium and pancuronium after 0.1 mg/kg intravenously, in man. Vertical bars bisecting the plasma curves indicate plasma concentrations at the time point of 50% recovery of muscle contraction. Modified from Sohn *et al.* (51).

the amine centers (see Fig. 4). The higher molecular weight threshold for bivalent cations in hepatobiliary transport, as compared to the monovalent cations, might reflect the amount of apolar elements in the molecule that is in general required to balance the bivalent ionic character. This may hold especially for the canalicular excretion, which occurs against the inside negative membrane potential. Thus the lipophilicity-hydrophilicity balance of the type II organic cations appears to be an important factor determining hepatic transport both at the uptake and secretion levels. However, some remarkable exceptions exist. For instance, stercuronium is rapidly taken up by the liver in spite of a small octanol/Krebs partition coefficient (44,69). In addition, the low molecular weight hydrophilic bivalent cations decamethonium and hexamethonium appear to accumulate in the liver to a substantial degree (70), whereas biliary output of these compounds is minimal (14,70). It is possible that for such compounds an endocytotic uptake mechanism is an alternative route. Such a process was recently described for the diquaternary fluorescent dye lucigenin that by virtue of its concentrated positive charges may induce absorptive endocytosis. This process leads to an endosomal/lysosomal sequestration without appreciable biliary output (71).

The hepatobiliary transport of several structurally related but physicochemically distinct steroidal muscle relaxants (Fig. 5) was recently studied in isolated perfused rat liver (72). Marked differences were observed in the overall hepatobiliary transport of the four muscle relaxants under study. The most hydrophilic compound, pancuronium, showed the lowest net transport from perfusate to bile. Conversely, the most lipophilic relaxant, vecuronium (the monoquaternary analog of pancuronium) showed the most efficient hepatobiliary transport. Vecuronium was very effectively taken up in the liver and within 2 hrs more than 60% of the dose was excreted in



	X	R_1	R_2	MW_{cation}	Oct./Krebs part. coeff.
Vecuronium	N	$OCOCH_3$	$OCOCH_3$	557	2.56
3-Hydroxyvecuronium	N	$OCOCH_3$	OH	515	0.032
Org 6368	N^+-CH_3	H	$OCOCH_3$	514	0.0145
Pancuronium	N^+-CH_3	$OCOCH_3$	$OCOCH_3$	572	0.0033

Fig. 5. Structural formulas and physicochemical data of some steroidal organic cations (peripheral muscle relaxants).

bile. The bisquaternary relaxants Org 6368 and 3-hydroxyvecuronium showed an intermediate behavior, with effective uptake in the liver but only modest excretion in bile. Similar to pancuronium, the amount in the liver versus time curves indicated that hepatic storage in a deep compartment might be involved in the hepatic disposition of steroidal muscle relaxants.

Cardiac glycosides are potent inhibitors of the hepatobiliary transport of bivalent organic cations (8,17,24,73). Hepatic uptake of the three relatively hydrophilic muscle relaxants was also strongly reduced by K-strophanthoside, whereas the uptake of the more lipophilic vecuronium was only partly affected at the dose ratio used. Similar to vecuronium, a modest effect of K-strophanthoside was also observed in studies with the rather lipophilic bivalent cation hexafluronium (73). This may imply that passive diffusion is partly involved in the hepatic uptake of these lipophilic muscle relaxants. Examples of other extremely lipophilic cations are methyldeptropine (8,76) and rhodamine B (74). The latter compound has been shown to enter the liver parenchymal cells by passive noncarrier-mediated diffusion in spite of the presence of a cationic center (75). Alternatively, the affinity of such relatively lipophilic cations for the supposed common carrier site may be so high that the cardiac glycoside fails to compete successfully during hepatic uptake.

PHARMACOKINETIC MODELING AND ANALYSIS OF HEPATIC ORGANIC CATION TRANSPORT

The plasma disappearance and biliary excretion rate versus time curves obtained after a single injection of 1 mg of the steroidal muscle relaxants in the perfusion medium of isolated perfused rat livers were fitted with the computer program DIFFIT. This program enables simultaneous fitting of plasma disappearance and biliary excretion rate curves, yielding the best model to explain these independently measured profiles. In contrast with the traditional nonlinear curve-fitting methods, this method is based on defining input and output of the drug, and subsequent simulation of disappearance and appearance patterns by a numerical approach. Iterative changing of the parameters, such as rate constants and volumes of distribution, is followed by comparison of the experimental data with the calculated curves, using least squares regression analysis. The combination of rate constants and distribution volume in the various differential equations were adapted until a minimal sum of squares was attained, using the Simplex procedure for an optimal simultaneous change of parameters. The program offers the possibility to test several multicompartment models without the necessity of deriving the complex equations by integration of sets of differential equations, as performed in the traditional compartment analysis.

Apparent volumes of distribution V_2 and V_3 were estimated from the amounts in the various compartments under simulated steady state conditons. After the fitting procedure the optimal model is discriminated by application of the F-ratio test. In the fitting procedure corrections were applied for loss of drug and circulating volume by sampling of perfusate. Fitting of experimental versus simulated curves was assumed to be optimal when the change in sum of squares was less than 0.01%. The experimental data were weighted according to the y^{-2}-method. The program was developed in cooperation with Zernicke Science Park, University of Groningen, as part of the software package "MW PHARM".

A kinetic analysis revealed that the hepatobiliary transport of the steroidal muscle relaxants was best described by a three-compartment model with elimination from the peripheral compartment V_2 and storage in a deep compartment V_3, connected to V_2 (see Fig. 6). Rather than molecular weight (12), the lipophilicity of the muscle relaxants influenced the kinetic parameters of the hepatobiliary transport. The biliary clearance (Cl_{20}) and the initial hepatic uptake (Cl_{12}) showed a positive relationship with the lipopholicity, confirming that hepatobiliary transport of these organic cations is highly dependent on the hydrophobic character of the compounds (8,10,11). In addition, net hepatic uptake of the muscle relaxants (rate of distribution to the liver) is markedly influenced by the liver to plasma transport (hepatic efflux). The tendency for hepatic efflux, expressed as the k_{21} value, increases with decreasing lipophilicity. This phenomenon can be envisoned if the dissociation of the relatively lipophilic compounds from

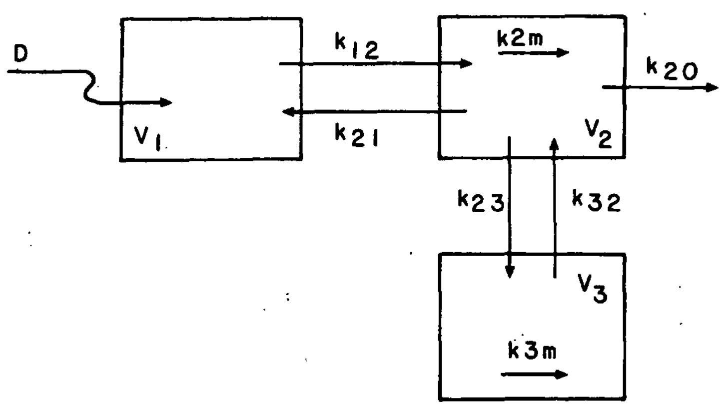


Fig. 6. Compartmental model for pharmacokinetic analysis of the hepatic uptake, storage, and biliary excretion of organic cationic drugs. Rate constants for transport between the compartments are indicated (fraction per unit of time), k_{2m} and k_{3m} represent rate constants for metabolism (de-acetylation) in compartment 2 and 3, respectively.

the carrier sites at the outer side of the membrane were the rate-limiting step in the hepatobiliary efflux process.

In analogy with the classical muscle relaxant d-tubocurarine (66,77–79) the kinetic analysis revealed an intracellular deep compartment. The affinity of the muscle relaxants for this intracellular distribution compartment, expressed as the k_{23}/k_{32} ratio, is inversely related with lipophilicity. However, it should be realized that the absolute amount of drug that is accumulating in the deep compartment is also determined by the fraction of the dose that can penetrate into the hepatocyte (80,81). The size of this fraction is determined by the k_{12}/k_{21} ratio, a factor that increases with increasing lipophilicity. Although the combined data indicate that lipophilicity has a major influence on several stages of the hepatobiliary transport of these organic cations, it is important to note that the net uptake of the muscle relaxants (indicated by the k_{12}/k_{21} ratio) is of special importance, inasmuch as the rate of the various subsequent transport steps is dependent on the concentration of the drug in the hepatocyte cytoplasm as the driving force for these processes.

HEPATIC TRANSPORT MECHANISMS FOR MONOVALENT ORGANIC CATIONS

Two compounds have been used most frequently as model compounds in the study of hepatobiliary transport of organic cations: procainamide ethobromide (PAEB) and its N^4-acetyl derivative (APAEB). Experiments with these compounds have been performed using several techniques such as rats *in vivo* (9,16–18,21,23,24,82), isolated perfused livers (17,30), rat liver slices (16,20,22), isolated hepatocytes (15,20,83), and liver membrane vesicles (84). An important contribution to a better understanding of hepatic transport mechanisms for organic actions was made by Schanker and co-workers. They showed that after intravenous injection of PAEB into the rat, the drug reaches concentrations in the bile far exceeding the concomitant plasma values (26). The biliary excretion was saturable and could be suppressed by structurally related compounds (21). Uptake into liver slices was blocked by anoxia and a number of metabolic inhibitors (22). These combined data indicated that carrier-mediated mechanisms are involved in hepatobiliary transport of organic cations, as was later on supported by other studies (16,19). Liver fractionation studies (23,24,30) pointed to two concentrative steps in the transport from blood to bile (8), indicating that energy-requiring transport systems are involved. Other mechanisms than passive distribution should be involved since concentration ratios of free PAEB between cytosol and plasma and between bile and cytosol considerably exceed the equilibrium value that is anticipated if distribution had

taken place according to the transmembrane potential (23,24). For the hepatic uptake step this was corroborated in a study with freshly isolated rat hepatocytes, which indicated that PAEB is taken up by a primary or secondary active carrier-mediated mechanism (15).

A new approach to carrier identification was introduced by the application of the technique of photoaffinity labeling in the field of hepatic transport. The concept of photoaffinity labeling (Fig. 7) is based on ligands that have an inherent affinity for a binding site but also contain a photosensitive functional group that, when photoactivated, is capable of forming a covalent bond at or near the binding site (85–91). Subsequent identification of the binding polypeptide is possible by concomitant introduction of a radioactive marker in the photolabile substrate. Unlike electrophilic affinity labels, their association with the recognition site will ordinarily be reversible until photolysis is initiated. The recent introduction of high-energy flash photolysis thus might enable investigations of time-dependent transport processes (86).

In order to identify potential transport polypeptides for these low molecular weight cations, a photolabile derivative of the classical model compound PAEB was synthesized: azidoprocainamide methoiodide (APM). Determination of the octanol/Krebs partition coefficient and binding to albumin of APM revealed that the introduction of the photolabile azido group did not markedly change the physicochemical parameters as compared to its parent compound PAEB. Experiments in isolated perfused rat liver demonstrated that APM is efficiently taken up in the liver and excreted into bile, partly in the form of metabolites (87).

The inhibitory effect of other cations such as tributylmethylammonium on the hepatobiliary transport of APM suggests that carrier-mediated

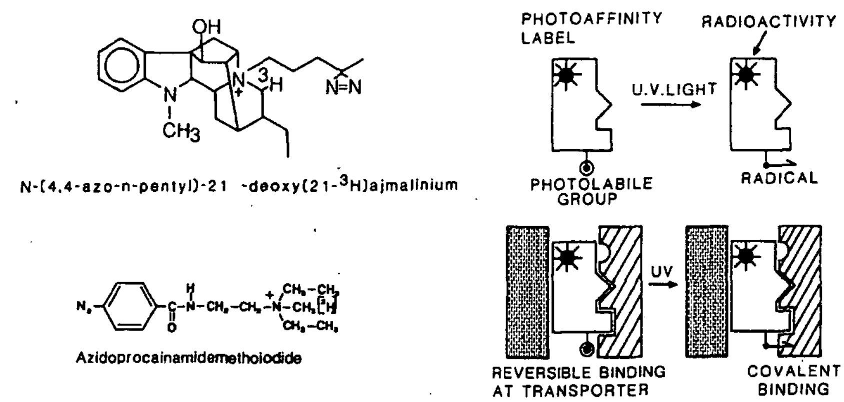


Fig. 7. Principle of photoaffinity labeling and photoaffinity probes of organic cations to label potential carrier proteins in hepatocyte plasma membranes.

mechanisms are involved, as was demonstrated for PAEB (15-17,20,22). Kinetics of the uptake of APM in isolated rat hepatocytes revealed that both saturable and nonsaturable processes are involved. The saturable part of the uptake was best described by two uptake systems ($V_{max1} =$ 80 pmol/min $\cdot$ 10^6 cells, $K_{m1} = 3\ \mu M$ and $V_{max2} = 130$ pmol/min $\cdot$ 10^6 cells, $K_{m2} = 100\ \mu M$). APM uptake was Na^+-independent, but replacement of bicarbonate in the incubation medium by chloride decreased the hepatic uptake. The mechanism of this bicarbonate effect, that occurs at an unchanged extracellular *p*H, remains to be clarified. An effect on secondary mechanisms involved in the driving of the uptake may underly the observed phenomenon.

In order to investigate the specificity of the hepatic uptake systems for APM, the influence of several classes of hepatic model compounds on the uptake of APM was studied. The results show that all the mono- and bivalent organic cations studied substantially decreased the uptake of APM into the liver cell. In presence of anionic and uncharged compounds the uptake of APM was not inhibited. These data indicate that APM is taken up by transport systems differing from the systems for anions and uncharged compounds. The complete lack of effect of high concentration of APM on the uptake of bulky organic cations indicates that APM is taken up by a system distinct from the uptake mechanisms for such agents, but that on the other hand the high molecular weight (Type II) compounds exhibit considerably affinity for the supposed uptake system for low molecular weight organic cations. Probably only carrier occupation without net transport occurs.

HEPATIC TRANSPORT MECHANISMS FOR BIVALENT ORGANIC CATIONS

Hepatobiliary transport mechanisms for bivalent cations have predominantly been studied using the classic muscle relaxant d-tubocurarine as the model compound. Besides the chemical difference in the number of cationic centers in the molecule, bivalent cations also show a marked difference in hepatobiliary transport, as compared with the monovalent cations. In the case of the bivalent cations, the hepatic uptake can be strongly inhibited with relatively low concentrations (1 μM) of cardiac glycosides (8,17,73). The biliary excretion step however seems not to be affected by these agents (73). In contrast, uptake of monovalent cations is not influenced by cardiac glycosides (15,17). The effect of the cardiac glycosides does not appear to be linked directly to their inhibitory effect on the Na^+K^+-ATPase, since the concentrations in which they inhibit the uptake of bivalent cations are too low to affect the Na^+K^+-ATPase of rat liver to a significant degree

(19,93). In addition K-strophanthoside and digitoxin are much more potent inhibitors of bivalent cation uptake than ouabain, while their potency to inhibit this ATPase is quite similar (94). The mechanism of uptake of steroidal cations has been investigated in isolated rat hepatocytes (80), using vecuronium as a model compound (81). Determination of initial uptake velocity at different vecuronium concentrations demonstrated that vecuronium uptake into the hepatocyte occurs by both a saturable ($K_m = 15\,\mu M$, $V_{max} = 181$ pmol/min $\cdot 10^6$ cells and a nonsaturable ($k = 1.10$ pmol/min $\cdot 10^6$ cells $\cdot \mu M$) process. This implies that in the *in vitro* situation the saturable component is mainly responsible for the hepatic uptake, since plasma concentrations in the rat usually do not exceed 5 μM.

The uptake of vecuronium satisfies the other criteria for carrier-mediated transport. The uptake is inhibited by structurally related compounds, is temperature-dependent, and decreased by various metabolic inhibitors. The inhibitory effect of SH-reagents indicate that sulfhydryl groups may be located at the active site of the transport system. Uptake into the liver of d-tubocurarine and metocurine is probably the rate-limiting step in the overall hepatobiliary elimination process as indicated by the low intracellular (cytoplasmic) concentration. However, this is not a general rule for the bivalent cations.

Determination of the unbound fractions of some cationic steroidal muscle relaxants in Krebs-albumin solution, in the cytosolic fraction of liver homogenate, and in bile, enabled a rough estimation of the concentration gradients across the sinusoidal and the canalicular membrane. The data indicated that major differences exist in the net sinusoidal uptake of pancuronium, Org 6368, and vecuronium which increased in the order pancuronium < Org 6368 < vecuronium. The calculated chemical gradients for vecuronium and Org 6368 were 16 and 1.6, respectively, and that for pancuronium less than 1 (see Fig. 8). Yet the cytosol-plasma concentration ratios of these agents did not exceed the values that would be attained by passive equilibration according to the membrane potential. At the canalicular site "uphill" transport of the muscle relaxants into bile against an electrochemical gradient occurs. Thus some kind of active transport is inferred, which appears to be more efficient for Org 6368 and vecuronium than for pancuronium. The nearly equal bile-cytosol concentration ratios for Org 6368 and vecuronium suggest that the differences in biliary excretion rate between these two substances are not caused by differences in membrane transport at the canalicular level, but rather by an unequal extent of binding within the liver cells. Since vecuronium and Org 6368 strongly differ in lipophilicity, it is evident that even a modest hydrophobicity is sufficient for effective canalicular transport of organic cations, which is in line with data of Neef *et al.* (35). The limited hepatobiliary transport of pancuronium

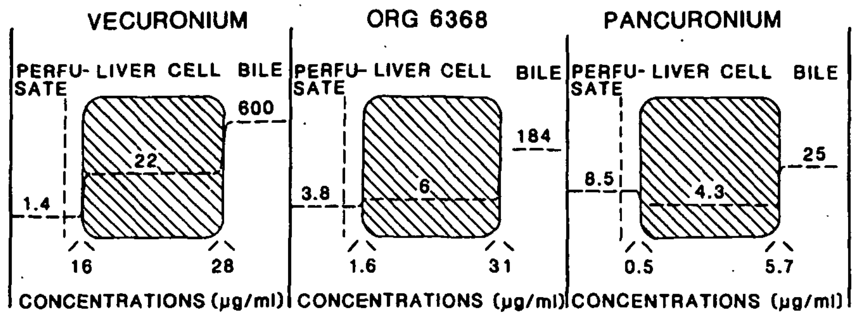


Fig. 8. Hepatobiliary concentration gradients of the Type II organic cations in rat liver. The unbound concentrations in perfusate, hepatic cytosol, and bile are indicated together with the calculated concentration gradients across the sinusoidal and canalicular membranes (81).

however appears to be due to inefficient net transport, both at the sinusoidal and at the canalicular membrane and apparently its hydrophobicity is below a critical level for proper carrier-mediated transport at these levels.

SUBCELLULAR DISTRIBUTION OF ORGANIC CATIONS IN RELATION TO HEPATIC STORAGE AND EXCRETION RATE

Accumulation in the liver may, apart from efficient membrane transport, also be explained by extensive intracellular binding (8,23,24,71). Quaternary agents are appreciably bound to the particulate fraction after liver homogenization (23,24,66). Liver subfractionation studies (10) as well as electron microscopy of d-tubocurarine molybdate precipitates in liver sections strongly suggest association with lysosomes in hepatocytes (66,78,79) (Fig. 9). The accumulation in these organelles can be partially inhibited by chloroquine (79). Association of organic cations with lysosomes presents a likely explanation for the persistent hepatic storage of these compounds in other studies (28,44,77,95). The mechanism of the lysosomal accumulation of this type of cation remains to be elucidated. Proton-driven antiport of organic cations from the cytosol into the lysosome, followed by intralysosomal trapping caused by the protonation of the tertiary amine group of the drug in this acidic compartment, might play a role (96). Triggering by organic cations of aspecific fluid phase endocytosis and subsequent vesicular transport to the lysosomes might form an alternative explanation (71). This mechanism has been proposed for the renal accumulation of the strongly basic aminoglycosides (97,98). Receptor-mediated co-endocytosis by noncovalent binding of d-tubocurarine to α_1-acid glycoprotein was recently excluded as a possible mechanism for lysosomal accumulation (99).

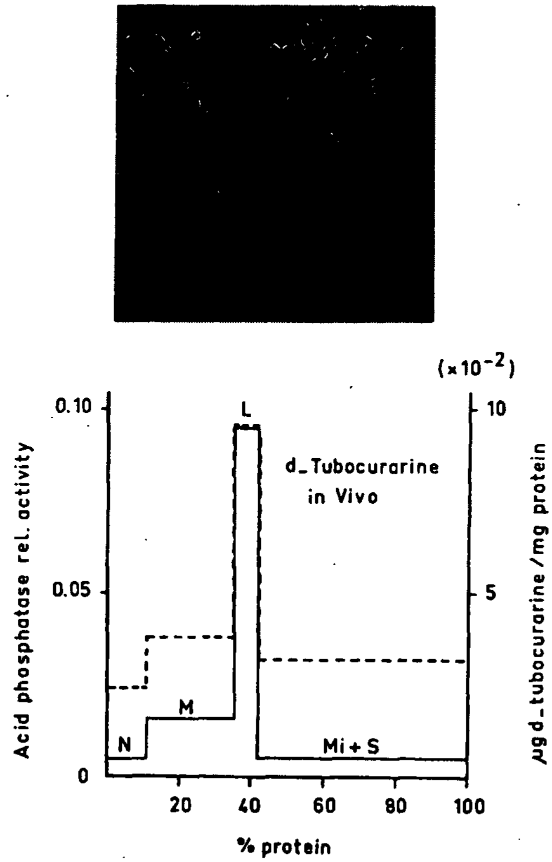


Fig. 9. Lower part: Subcellular distribution of d-tubocurarine and acid phosphatase in the liver 15 min after iv injection of 2 mg/kg of ^{3}H-d-TC into rats (*in vivo*) and after the addition of ^{3}H-d-TC to cold liver homogenates (*in vitro*). Specific activities are indicated ($n = 3$). The broken line indicates distribution of d-TC and the solid line that of acid phosphatase. Fractions are represented by their protein content cumulatively from left to right in the order of their isolation. N, M, L, and S indicate the nuclear, mitochondrial, lysosomal, and supernatant fraction, respectively. Upper part: Electronmicroscopy of liver sections of d-tubocurarine-treated rats. After fixation the liver tissue blocks were treated with ammonium molybdate, a substance that gives an electron-dense precipitate with curare-like drugs. Small electron-dense particles are found in and around lysosome-like bodies in the hepatic parenchymal cells. The dense deposits are not found in livers from untreated control animals.

Pancuronium, Org 6368, and vecuronium liver homogenate subfractionation (100) indicated that the steroidal muscle relaxants were predominantly accumulated in the mitochondrial fraction (95). Accumulation in mitochondria has been earlier demonstrated for various other mono- and bivalent cations (47,101–103) and might be explained by passive equilibration according to the mitochondrial membrane potential (47). Alternatively, active carrier-mediated uptake of organic cations in mitochondria might be involved (103). In this context a potential interaction with the endogenous substrate NAD^+ has been inferred (101–103). In comparison with the classical muscle relaxants d-tubocurarine and metocurarine (66,78,79) accumulation in the lysosomal fraction seemed to be of less importance in the case of the steroidal muscle relaxants and decreased relative to the association with the mitochondrial fraction if the liver load was increased. The particle–cytosol concentration ratio of Org 6368 was significantly higher than the values for vecuronium and pancuronium and might explain the effective intracellular storage of Org 6368, as observed in the isolated perfused rat liver experiments (69) as well as in cat *in vivo* (49).

MULTIPLICITY IN HEPATIC UPTAKE MECHANISMS FOR DRUGS

The classical view on hepatic uptake mechanisms states that there are three separate transport pathways, depending on the charge of the substrate (anions, cations, and uncharged compounds) (5,7,104). For several reasons this traditional scheme may require revision. As discussed above, the effect of cardiac glycosides on the uptake of cations discloses a marked difference in uptake between bivalent and monovalent cations. Similarly, for organic anions at least two uptake systems are inferred (105–107). With respect to organic anions one roughly differentiates between sodium-independent (bilirubin, BSP) and sodium-dependent (bile acids, fatty acids) uptake systems. Yet also for bile salts and for fatty acids, which are both taken up by sodium-dependent mechanisms, separate systems are demonstrated (108), indicating that more than two carrier-mediated systems are involved in the uptake of organic anions (92). It is conceivable that the diversity in organic anion uptake systems is related to the presence of various naturally occurring anionic substrates such as bile salts, bilirubin, and fatty acids. An analogous situation appears to hold for the uncharged compounds, which include many naturally occurring substrates such as steroid hormones. Data from uptake studies with various uncharged compounds (cardiac glycosides, steroid hormones) suggest the existence of more than one uptake system for uncharged compounds (109,110). These combined data indicate

that the liver is equipped with multiple uptake systems with limited substrate specificity.

On the other hand the concept of separate uptake pathways for organic anions, cations, and uncharged compounds is challenged by the occurrence of major interactions between representatives of these classes of compounds. Traditionally, many studies on hepatic transport dealt with bile salts. Kinetic data show that bile salts can not only inhibit the hepatic uptake of other organic anions (107,111–114) but also of the uncharged cardiac glycosides (110,115,116), cyclopeptides (112,117,118), and bivalent cations (29,69). High bile salt plasma concentrations have been shown to prolong the effect of muscle relaxants, both in animal and man (66,119), most probably due

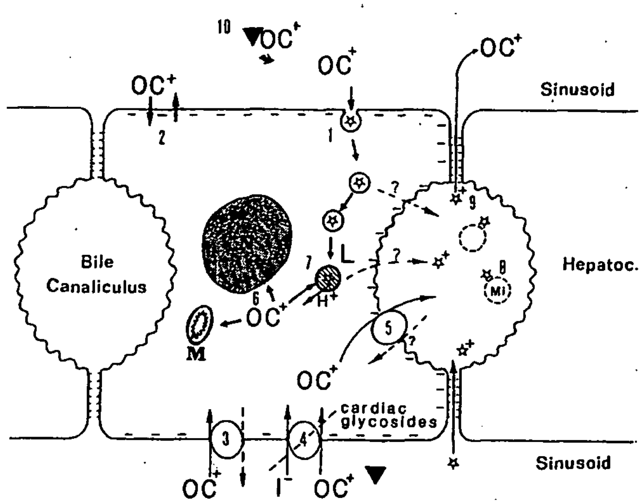


Fig. 10. Mechanisms for uptake of organic cations into hepatocytes. Adsorptive endocytosis (1) leads to accumulation of the cation in endosomes, lysosomes and other vacuoles. Lipophilic cations can also enter the cell by passive diffusion (2), others undergo carrier-mediated uptake. At least two carrier systems are involved: one for small cations (3) and one for larger cationic compounds (4). At least one carrier system mediates biliary excretion of organic cations (5). Direct uptake from the cytoplasm takes place into nuclei (6), into mitochondria and into lysosomes (7). Accumulation in lysosomes may, apart from aspecific fluid-phase endocytosis at the plasma membrane, occur from the cytoplasm via antiport with protons within the lysosomes. Direct transport of drug from lysosomes to bile could occur extremely slowly by exocytosis. Biliary excretion involves carrier-mediated transport, possibly by antiport with inorganic ions. Binding to mixed biliary micelles may facilitate net transport into the bile canaliculus (8). Small organic cations can probably directly pass the tight junctional channels that have a negative charge (9). Intrahepatic dissociation from α_1-acid glycoprotein (triangle) occurs in the sinusoids (10).

to uptake inhibition of the muscle relaxant by the bile salts (69). Also the reverse inhibition has been reported: The uptake of bile salts in the hepatocyte can be decreased in presence of steroid hormones (113,120), cardiac glycosides (105,121), cyclopeptides (112,122), cholecystographic agents (112), and various other compounds (123–125). Furthermore, mutual uptake inhibition between representatives of these different groups of substrates has been reported (15,78,115,116,122,125–127,131). Cardiac glycosides inhibit the hepatic uptake of bivalent cations (17,73) and bivalent cations have been shown to decrease the uptake of ouabain into isolated hepatocytes (115). The occurrence of these mutual interactions might be explained by the existence of uptake systems with broad substrate specificity (88,115,125,128). A general transport system for bulky organic compounds with multiple ring structures, irrespective of charge, presents an attractive explanation for the observed interactions (1,2,88,129). The detoxification function of the liver may benefit from such a system with broad substrate specificity.

In conclusion, based on the results of the aforementioned studies, the hepatic disposition of organic cations can be schematically pictured as depicted in Fig. 10.

IDENTIFICATION OF POTENTIAL CARRIER PROTEINS

In addition to kinetic studies, attempts were made to identify potential transport polypeptides for several categories of drugs (Fig. 11). Table I summarizes studies on identification of potential carrier proteins in rat liver, using various affinity labeling techniques. From this table it is evident that the majority of the identified binding polypeptides for the different model compounds have apparent molecular weights of about 48,000 and/or 55,000, irrespective of the technique used. In combination with kinetic data the results of these studies have been interpreted as supporting the hypothesis of uptake systems with broad substrate specificity (91,129). With respect to the identification of carrier polypeptide(s) for uncharged compounds such as cardiac glycosides and organic cations, only limited information is available. A M_r 50,000 polypeptide was labeled with a photolabile ouabain derivative (90). Since ouabain is taken up in the liver cell by a Na^+-independent carrier-mediated mechanism (115,130,131), the labeled polypeptide would be anticipated to represent this Na^+-independent system. Whether or not the M_r 50,000 binding polypeptide described by Petzinger *et al.* (90) is the same as the M_r 48,000 or 54,000 protein that is labeled with bile salt derivatives, remains to be established. It is tempting to relate the two major labeled proteins to the Na^+-dependent and Na^+-independent transport systems for organic anions. Bilirubin and BSP, that are taken up

POTENTIAL CARRIER-PROTEINS FOR HEPATIC TRANSPORT OF DRUGS

Mol Weight	48 kDa	48–49 kDa	50 kDa	54–55 kDa (OABP)	54–55 kDa	55 kDa	100 kDa	110 kD (BTL, $\alpha_2\beta$)
Protein-isolation	—	+	–	+	–	+	+	+
Photo-aff, label	+	+	+	+	+	–	+	–
Antibody	–	+	–	+	–	+	+	+
Reconstitution	–	–	–	–	–	–	+	+
Co-factors	H^+	Na^+	HCO_3^-	CL^-	OH^-	–	HCO_3^-	H^+ ?
Sinusoidal/Canalicular	S	S	S	S	S	S	C	S
References	1,87,88, 89,146	89,90,91, 129,133, 135,136, 141,168, 173,174	1,88, 89,146	139,172	88,90, 91,117, 147,162, 173,174, 175	92,138, 170,171	143,144, 163	137,140, 169
Substrates	Organic Cations (Type I)	Bile Acids Phalloidin	Organic Cations (Type II)	BSP (GSH) Bilirubin	BSP Bile Acids Steroids	BSP (GSH) Bilirubin ICG	Bile Acids	BSP Bilirubin ICG Rifamycin Nicotinic Acid

Fig. 11. Experimental work reported on potential carrier proteins responsible for hepatic and biliary excretion of organic anions and other drugs. Estimated molecular weight, use of photoaffinity techniques, preparation of antibody with measurement of its effect on uptake, reconstitution experiments performed, possible cofactors or driving forces established, and binding or transport of substrates are indicated. The scheme suggests separate carrier processes; however, some may turn out to be identical (for instance, 1st, 2nd, 3rd and 4th, 5th and 6th from left to right).

by a Na^+-independent mechanism, appear to label predominantly a M_r 55,000 polypeptide, whereas bile acid derivatives label both the 48,000 and 55,000 species. Since mutual competitive inhibition has been demonstrated for BSP and Na^+-independent bile acid uptake (107,111), the M_r 55,000 polypeptide might be a likely candidate for the Na^+-independent BSP transporter. In this concept the polypeptide with apparent molecular weight of 48,000 might be related to the Na^+-dependent bile acid uptake system.

Photoaffinity labeling with the Type I organic cation azidoprocainamide methoiodide (APM) was performed on isolated hepatocytes and sinusoidal membrane fractions. The experiments revealed two membrane polypeptides with apparent molecular weight of 48,000 and 72,000 that are involved in binding with APM. Differential photoaffinity labeling in presence of substrates that inhibit the uptake of APM suggested that these polypeptides might be involved in the hepatic uptake of APM (87).

Photoaffinity labeling with a photolabile derivative of N-propyldeoxyajmalinium (NPDA), a bulky monovalent quaternary amine that was used as a Type 2 model compound was recently performed (146). Physicochemical parameters, such as albumin binding and lipophilicity (expressed as octanol/Krebs partition coefficient) of NPDA appeared to be highly comparable with the steroidal muscle relaxant vecuronium. Studies in isolated perfused rat liver demonstrated mutual uptake inhibition for both

Table I. Binding Polypeptides in Hepatocyte Plasma Membranes as Identified by Different Affinity Labeling techniques

Parent compound	App. MW ($\times 10^{-3}$)	Technique[a]	Preparations used: Intact cells	Plasma membranes	Authors (Reference no.)
Procaine	21.4	PAL	—	V	Levy *et al.*, 1977 (132)
BSP	60	AC	—	V	Reichen and Berk, 1979 (170)
	55	PAL	—	V	Wolkoff and Chung, 1980 (172)
	37/35		—	V	Lunazzi *et al.*, 1982 (169)
	56	AC	—	V	Stremmel *et al.*, 1983 (171)
Bilirubin	60	AC	—	V	Reichen and Berk, 1979 (170)
	56	AC	—	V	Stremmel *et al.*, 1983 (171)
	54/48	PAL	V	—	Buscher *et al.*, 1986 (88)
NAP-taurine	54/43	PAL	V	V	Cheng and Levy, 1980 (168)
	54	PAL	V	—	Von Dippe and Levy, 1983 (133)
Taurocholate	52/48	PAL	—	V	Kramer *et al.*, 1982 (147)
	54	PAL	V	V	Von Dippe and Levy, 1983 (133)
	54/48	PAL	V	V	Wieland *et al.*, 1984 (91)
Cholate	54/50	AL	—	V	Ziegler *et al.*, 1984 (173)
Taurodehydrocholate	54/50	AL	V	V	Ziegler *et al.*, 1984 (174)
DIDS	54/50	AL	V	V	Ziegler *et al.*, 1984 (174)
Phalloidin	54/48	PAL	V	V	Wieland *et al.*, 1984 (91)
Antamidine	54/48	PAL	V	V	Wieland *et al.*, 1984 (91)
Oleate	40	AC	—	V	Stremmel *et al.*, 1985 (108)
Ouabain	50	PAL	—	V	Petzinger *et al.*, 1986 (90)
α-Amanitin	54/48	PAL	V	V	Kröncke *et al.*, 1986 (117)
Cyclosporine	54/50	PAL	V	V	Ziegler and Frimmer, 1986 (175)
Ajmalinium	48	PAL	V	—	Buscher *et al.*, 1986 (88)

[a]PAL: photoaffinity labeling; AL: affinity labeling; AC: affinity chromatography.

compounds. Furthermore the hepatic uptake of the monovalent cation NPDA was inhibited by taurocholate and by K-strophanthoside, thus showing a striking parallel with vecuronium-like compounds (147).

It is crucial to prove that the identified polypeptide not only binds the drug but that it is also able to translocate the substance across the membrane. Indirect approaches to relate photoaffinity labeling to transport function include comparison with photoaffinity labeling in cells that lack the transport function under study, e.g., AS 30 D hepatoma cells (134) or studying the rate of uptake after photoaffinity labeling (135). An alternative approach is to study the effect of antibodies, raised against the purified protein, on drug transport. Recently several groups reported inhibitory effects of antibody preparations on the uptake of organic anions (108,136–139). Stremmel and Berk (138) and Wolkoff *et al.* (139) described an antibody against a BSP/bilirubin binding protein with apparent molecular weight of 55,000. The antibody prepared by Stremmel and Berk inhibited uptake of BSP and

bilirubin but did not affect the uptake of taurocholate, cholate or oleate in isolated hepatocytes (138). Since the antibodies of both groups showed only weak cross-reactivity, further comparative studies are necessary to elucidate structural and immunological similarities. Levy *et al.* (136) reported an antibody against a M_r 54,000 membrane protein, which specifically inhibited Na^+-dependent taurocholate transport in isolated hepatocytes but had no effect on BSP transport. Integration of these data suggests that at least two proteins with apparent molecular weights in the range of 55,000 are involved in organic anion transport (92).

Besides immunological studies, reconstitution of transport with the isolated proteins might provide evidence for their physiological function. Sottocasa *et al.* (140) performed reconstitution experiments with the potential carrier protein bilitranslocase. The results showed transport of BSP, which could be driven by a negative membrane potential, a process that could be imagined in relation to sinusoidal or canalicular secretion from the cell. Von Dippe *et al.* (141) reported reconstitution in liposomes with a M_r 54,000 polypeptide that exhibited several characteristics of Na^+-dependent taurocholate transport: However, substrate specificity of the reconstituted transport systems was not investigated in either study.

Only limited data are available with respect to the polypeptides involved in bile-canalicular transport. By photoaffinity labeling of rat liver snips, a preparation in which functional polarity is preserved, Fricker *et al.* (142) identified a bile salt-binding polypeptide with an apparent molecular weight of 100,000 at the canalicular membrane. Inhibition of bile salt transport in canalicular plasma membrane vesicles by an antibody against this M_r 100,000 polypeptide indicated that this protein is involved in canalicular excretion of bile salts (143). Transport could be reconstituted in liposomes (144) and was shown to be basically different from the sinusoidal Na^+-dependent uptake system.

With regard to potential canalicular transport polypeptides for organic cations, Kamimoto *et al.* (145) reported data on a canaliculnr membrane protein with an apparent molecular weight of 170,000, which might be the efflux pump for amphipathic cationic anticancer drugs in hepatocytes. The membrane protein has two internal ATP binding sites, and ATP-dependent transport of anticancer drugs in canalicular membrane vesicles was inhibited by verapamil and other drugs. More data are available on membrane proteins that are involved in the hepatic uptake process for amphipathic cationic drugs.

Subsequent photoaffinity labeling with the photolabile NPDA-derivative of isolated cells revealed two plasma membrane binding polypeptides with apparent M_rs of 48,000 and 50,000. Differential photoaffinity labeling studies demonstrated decreased incorporation of radioactivity in the plasma membrane polypeptides in the presence of amphipathic cations

(vecuronium, pancuronium, d-tubocurarine, quinidine, and verapamil) and in the presence of uncharged compounds (K-stophanthoside, ouabain, digitoxin), compounds that all inhibit the uptake of NPDA in cells. These data suggest that the identified binding polypeptides may be involved in the hepatic uptake of the amphipathic cation (88, 146).

DRIVING FORCES IN THE HEPATIC TRANSPORT OF DRUGS

The general idea of uphill transport raises the question on the energization and driving forces for these processes. In the case of taurocholate numerous studies indicated that the carrier-mediated hepatocellular uptake is driven by an inwardly directed Na^+-gradient (105,111,124,148–150). The process is characteristic of a Na^+-coupled secondary active transport driven by the Na^+-gradient that is maintained by the activity of the basolateral Na^+K^+-ATPase. Several studies with basolateral membrane vesicles showed facilitation of taurocholate uptake by the negative intracellular potential, suggesting that cotransport occurs with more than one Na^+ ion (142,148–151). Recent data suggest that only certain conjugated bile acids are transported by this system (154) and alternative forms of uptake may be important for other types of bile acids (105,124,153). Cholate transport is dependent upon the presence of chloride, which might be selectively needed as a discharging counteranion (155). Data from a study of Blitzer *et al.* (156) suggest hydroxyl/cholate exchange related to the Na^+/H^+ exchanger that has been identified in the basolateral membrane (157). The uphill cholate transport might be explained by a "tertiary active transport" model in which the Na^+K^+-ATPase would ultimately drive the Na^+/H^+ exchange, which in turn would drive hydroxyl/cholate exchange (156). Alternatively, the results can also be explained by H^+/cholate cotransport, and may therefore in principle occur by passive noncarrier-mediated diffusion of the unchanged protonated molecule.

Hepatic uptake of anions like BSP and iodipamide occurs by Na^+-independent mechanisms. In the uptake of both compounds an electrogenic component might be involved and appears to be related to an inwardly directed chloride gradient (130,158–160). Analogous chloride dependency was demonstrated in the carrier-mediated uptake of *p*-aminohippurate in rat renal basolateral membrane vesicles (161). In a study with short-term cultured rat hepatocytes a mechanism involving Cl^-/organic anion exchange was proposed (160). Studies with vesicles of sinusoidal and canalicular membranes revealed exchange mechanisms with inorganic anions (sulfate, bicarbonate, chloride) that appear to be involved in hepatobiliary transport of xenobiotics (162,163). The opportunities would provide a flexible and dynamic combination of transporting systems for organic compounds.

Information on potential driving forces for organic cation transport is scarce. Even with respect to possible Na^+-dependency of organic cation uptake, no clear picture emerges from the available data. Studies on the uptake of morphine, nalorphine (164), and thiamine (23) suggest that this process is partly Na^+-dependent, whereas the uptake of PAEB appears to be Na^+-independent (15). The inside negative membrane potential might provide the driving force for the uptake of organic cations by facilitated diffusion, a mechanism that has also been proposed for the organic cation transport across the basolateral membrane in the kidney (3,31). The uptake of vecuronium also appears to be independent of the Na^+-concentration in the extracellular medium. On the contrary the uptake of vecuronium exhibits anion dependency. Both cotransport of the cationic substrate with inorganic anions to maintain electroneutrality (44,89,165) and carrier-mediated uptake of (electroneutral) ion pairs (1,2,86,153,166) with vecuronium, formed at the fluid/lipid interface, might explain the observed anion dependency. In sucrose medium, a medium that increases the negative transmembrane

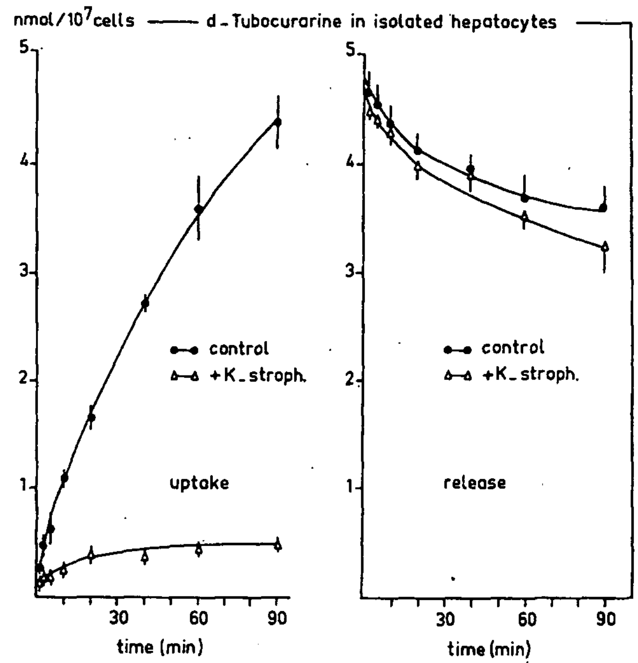


Fig. 12. Uptake of d-tubocurarine during incubation with isolated hepatocytes that can be strongly inhibited by K-strophanthoside at a concentration of 10^{-5}M. In contrast (right) the cardiacglycoside in the same concentration does not affect release from hepatocytes preloaded with d-tubocurarine.

potential, the uptake of vecuronium was decreased instead of increased, supporting the concept of electroneutral uptake of the muscle relaxant.

As discussed before, both kinetic and photoaffinity-labeling studies provided evidence that the liver is equipped with transport systems with broad overlapping substrate specificity (88,91,129). The effective inhibition of d-tubocurarine by cardiac glycosides (17,73) might therefore be related to a common uptake pathway for uncharged compounds including drugs with masked or neutralized charges (see Fig. 12).

The stimulating effect of ion pair-forming anions such as iodide on the hepatic uptake of organic cations also suggests transport as an electroneutral species (1,167). In the kidney organic cation transport across the brush border membrane occurs by a cation/proton antiport, resulting in net electroneutral uptake (31,118,166). Whether an analogous mechanism might

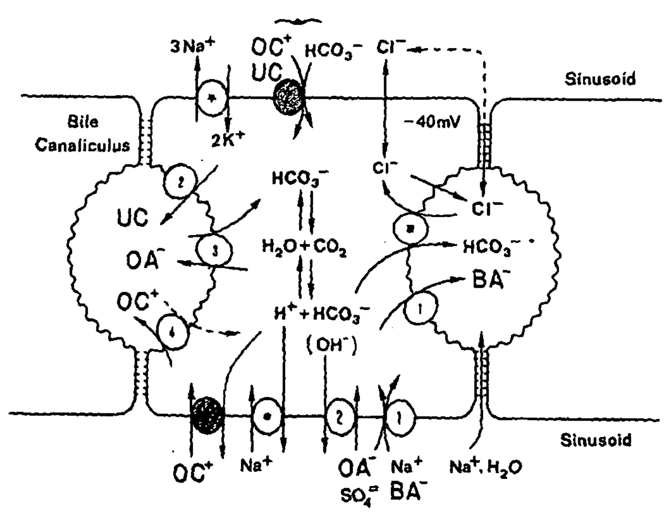


Fig. 13. Coupling between gradients of inorganic ions with carrier-mediated transport of organic anions (OA^-), bile acids (BA^-), organic cations (OC^+) and uncharged compounds (UC) in the hepatocyte. Inorganic ion-pumps include electrogenic Na^+/K^+ exchange, Na^+/H^+, OH^-/SO_4^{-2}-antiport (sinusoidal) and Cl^-/HCO_3^- and SO_4^{-2}/HCO_3^- antiport (canalicular). Na^+-coupled bile acid transport (1) and OH^-/OA^--antiport (2) at the sinusoidal level and HCO_3^-/OA^--antiport at the canaliculi are carrier systems for organic anions. Organic cations of Type I are taken up by System 1 and Type II compounds may share a transport System 2, also accommodating uncharged compounds. In the latter system ion pair formation may play a role. At the canalicular level antiport of organic cations with inorganic cations is tentatively assumed. The transport processes at sinusoidal level have more overlapping substrate specificity than the projected four canalicular carrier systems. [Adapted from G. Hugentobler and P. J. Meier (162).]